THERMAL INACTIVATION OF *BACILLUS ANTHRACIS* USING LASER IRRADIATION OF MICRO-ETCHED PLATFORMS

THESIS

Jeffrey B. Bacon, Major, USA

AFIT/GWM/ENP/09-M01

DEPARTMENT OF THE AIR FORCE
AIR UNIVERSITY

AIR FORCE INSTITUTE OF TECHNOLOGY

Wright-Patterson Air Force Base, Ohio

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AFIT/GWMD/ENP/09-M01

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THESIS

Presented to the Faculty

Department of Engineering Physics

Graduate School of Engineering and Management

Air Force Institute of Technology

Air University

Air Education and Training Command

In Partial Fulfillment of the Requirements for the

Degree of Master of Science in Combating Weapons of Mass Destruction

Jeffrey B. Bacon, MS

Major, USA

March 2009

AFIT/GWMD/ENP/09-M01

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Jeffrey B. Bacon, MS

Major, USA

Approved:

Larry Burggraf (Chairman)	9 Mar 2009 Date
Charles Bleckmann (Member)	9 Mar 09 Date
Guangming Li (Member)	9 Mar 2009 Date
William Baker (Member)	9 Mar 2009 Date
Daniel L. Felker (Member)	9 MAR 2009 Date

Abstract

The purpose of this research was to determine the probability of kill for a thermal inactivation strategy for use against biological agents; specifically the resilient endospore of *Bacillus anthracis* (*Ba*). The effort focused on short durations (milliseconds to several seconds) and temperatures (300 to 1300K) simulating the periphery effects after an explosion generated by conventional munitions. For an improved statistical counting, applied microlithography techniques were used to produce micro-etched glass platforms consisting of 532 circular sample wells, evenly spaced, with dimensions of 50μm in diameter and approximately 3μm in depth. Small carbon black radiators, which provide fast heating / cooling rate and confined temperature distribution, were produced by populating the etched wells with fine carbon particles of less than one μm for good contact with the spores. In order to prevent the carbon black from oxidation at high temperatures in air, a multifunctional sol-gel coating was designed to cover both the hydrophilic glass surface and hydrophobic carbon surface. *Ba* spores were sparely populated into the small wells on another micro-etched platform for improved statistical counting. The platform with carbon wells was paired with the other platform populated with the spores by aligning row by row and column by column using a laser diffraction method aided with an infrared beam finder.

The study refined techniques to populate the sample wells with as few as one *Ba* spore per well. This enables researchers to qualify, quantify, treat and measure small samples of spores over time. Spores were heated against black carbon wells using a solid

state laser (Nd: YAG). Heating temperatures were varied by using different laser powers. The heating times were controlled by adjusting the raster rate of the sample relative to the laser beam. The thermal radiation from the carbon well was measured using a Fourier-Transform Infrared (FTIR) spectrometer with a sensitive Mercury Cadmium Telluride (MCT) detector, from which the temperature was obtained by fitting a Planck function to the infrared spectrum from carbon. Samples were characterized before and after germination using optical microscopy and manual counting techniques. The statistical correlation between heating time, heating temperature, and spore viability was analyzed.

To my wife and children

Acknowledgments

I would like to express my sincere appreciation to my faculty advisor, Dr. Larry W. Burggraf, for his guidance, time and support throughout this research effort. The breadth of your knowledge regarding multiple disciplines is an inspiration, and motivator, for me as I continue my education in the future. I would also like to thank the remaining members of my research committee for their individual contributions to the work in their respective fields. Specifically, I'd like to thank Dr. Charles Bleckmann and Dr. Daniel Felker for serving as my sounding board and for their tireless efforts throughout. My sincere thanks goes to Dr. Guangming Li and Dr. William Baker, for their unique ability to contribute practical solutions to both academic and application problems alike. I would like to thank my sponsor Ms. Angelica Rubio, from the 709th Nuclear Systems Squadron, for her technical and fiscal contributions to the work. Finally, I'd like to thank my academic peers for their help and teamwork at the lab bench.

MAJ Jeffrey B. Bacon

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THERMAL INACTIVATION OF *BACILLUS ANTHRACIS* USING LASER IRRADIATION OF MICRO-ETCHED PLATFORMS

1. Introduction

1.1 Background

Biological weapons pose the most significant terrorist threat to the United States of America. The US Congressional Office of Technology Assessment estimated in a 1993 testimony before Congress that an aerosol release of just "100kg of *Bacillus anthracis* (*Ba*) spores upwind of Washington, D.C. would result in upwards of three million deaths". This casualty rate could be equated to those associated with the use of a hydrogen bomb (Inglesby, 2002). While acquiring a *Ba* weapon is much easier than acquiring a nuclear weapon, the detection, prevention and response to a biological attack would prove, arguably, much more difficult. Post attack diagnosis, antibiotic treatment, implementation of quarantine, and vaccination efforts could result in an unprecedented civil disruption. An actual, though smaller scale, example occurred shortly after the terrorist attacks of September 11, 2001, when four letters containing dried Ba spores were mailed to members of the United States Senate and two prominent news organizations. These letters can best be described as a point attack which targeted specific individuals or members of their staff. The mailings resulted in 22 confirmed or suspected cases of anthrax infection. Eleven of these were inhalational cases, the most severe, of which five

people died (Inglesby, 2002). Dr. Vahid Majidi, the Assistant Director responsible for the Federal Bureau of Investigation's (FBI's) Weapons of Mass Destruction Directorate, stated on August 6, 2008 that "we have developed a body of powerful evidence that allows us to conclude that we have identified the origin and the perpetrator of the 2001 *Bacillus anthracis* mailings" (U.S. Department of Justice, 2008). Forensic microbiology techniques led investigators to conclude that material used in the 2001 attacks genetically matched material from the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Maryland. Specifically, the investigation led to an Anthrax researcher, Dr. Bruce Ivins, who took his own life before charges were filed. Inferences in to Ivins' motivation ranged from monetary gain to a means of informing the United States government of the nation's vulnerability to a biological attack following the events of September 11, 2001. These acts resulted in death, disability and widespread fear regardless of the intentions of those responsible. Legislation and funding followed shortly thereafter and a marked increase in biological agent detection, decontamination, disease prevention, disease characterization and treatment research began. *Ba*, an established and prevalent biological agent studied since the 1800s, was brought back to the forefront of terrorist threats.

1.2 Purpose of the Research

Initiatives to evaluate weapon effectiveness in defeating biological and chemical weapons are ongoing and pending development. Tools to aid in the planning and assessment of weapons effects associated with offensive strikes on biological weapon facilities, stockpiles, and transport assets are needed. Weapons effects for chemical and

biological weapons are based on terms such as probability of kill which is defined as "achieving a defined level of agent neutralization for all of the agent stored in the target" (Defense Threat Reduction Agency, 1999). Other definitions and measures of effectiveness include damage to a defined percentage of containers, (ie: barrels, rockets, tanks) and the rendering of a dispersal and/or production devices unusable. Spilling as much of the material as possible onto the floor of the storage facility via gravity-driven leakage denies the adversary use of the CB weapons. Other factors considered with probability of kill analyses are secondary impacts such as collateral hazard prediction; in example container kick outs, unintentional release, and secondary plumes. The greatest health threat posed to nearby civilians is exposure to aerosolized chemical or biological agent. The goal is to maximize the number of containers damaged by the weapon while minimizing the amount of agent lofted in an aerosolized form. Temperatures and durations to simulate the environment within a conventional explosive's fireball to defeat a stockpile of anthrax spores would be valuable. This operational requirement has resulted in the development of new models for quantifying agent characteristics as well as the use of established weapon effects, transport and dispersion, and human effects prediction tools. One such model is the Simulation Environment & Response Program Execution Nesting Tool (SERPENT). SERPENT is designed to "accommodate end-to-end counter-CB target defeat analysis and 'real-world' probabilistic assessment" (Air Force Nuclear Weapons and Counterproliferation Agency, 2007). Several supporting models feed in to SERPENT to include an atmospheric dispersion model, an interior dispersion model, blast modeling and agent neutralization models. One contributing

model is the Empirical Lethality Methodology (ELM) model which predicts the probability of kill for biological and chemical agents. Inactivation is evaluated using Monte-Carlo tracer particle time histories. Each tracer is associated with the specific mass of agent, biological or chemical, with the resulting algorithms denoting inferred inactivation strategies; time and method. Confirmation and completion of existing data is essential to accurately predict neutralization in the planning of counterforce strikes using thermal fireball of conventional munitions on bio-weapon stockpiles. Inconsistent data exists for the thermal inactivation of *Ba* spores, with little data available for short duration exposures less than 30 seconds.

1.3 Problem Statement

Determine the probability of kill for *Bacillus anthracis* (*Ba*) spores using high heat, 300-1300K (27-1027°C), at short duration exposures, 0.01 to 3 sec.

1.4 Research Objectives

In order to characterize the thermal susceptibility of *Ba* spores for a range of temperatures and times, from milliseconds to several seconds, a laser heating method has been developed at AFIT to inactivate spores for a known temperature and exposure time. This laser heating method can expose spores to a near square temperature pulse with well controlled temperature and short heating time by rastering the laser across an absorbing surface at different rates (Goetz, 2005). The temperature – time profile was determined by measuring the Plankian emission spectrum from the heated surface using a Fourier-Transform Infrared (FTIR) interferometer. The microscopy method used previously to

measure the number of irradiated spores that germinated in time had disadvantages. One of these problems was that germinated *Ba* cells divide quickly and obscure the field of view, so that spores which germinate later were obscured. In order to separate spore colonies for microscopic counting MAJ L. Hawkins developed a technique for dispersing spores into microscopic holes etched in microscope slides which served as platforms for both laser irradiation and microscopic counting (Hawkins L. S., 2008). This technique also opened the opportunity to address a problem with the laser irradiation technique.

The laser irradiation method heated a refractory absorber, silicon carbide (SiC) adjacent to the spores that were adsorbed on the glass slides. Previous studies by Capt. K. Goetz (Goetz, 2005) showed that the emission spectrum from the heated SiC surface was composed of at least two temperatures, a high temperature corresponding to the surface under the laser spot and a lower temperature corresponding to conductive heating of the SiC surface nearby the laser spot. This emission from conductive heating reduced the precision for the temperature measurement and it exposed spores to a more gradual increase in temperature than the desired "top hat" exposure profile. This experimental research complements a companion computational study by Capt Emily Knight. This research is focused on improving the technique, eliminating the deficiencies and application of the technique to determine the influence of water content in the spore on inactivation probability:

- Address previously identified problem of measuring both spore growth and spore thermal kill threshold using the traditional method of spreading a diluted spore solution on a plate.

- Refine production of micro-etched platforms to facilitate spore separation. Intent is to prevent neighbor growth from obscuring germination and initial vegetative growth measurements.
- Refine characterization of the thermal heating environment. Model the sample configuration to determine heat loss between source temperature and that to which the spores are exposed.
- Determine the effects of environmental conditions, for example: desiccation, humidity, sporulation temperature, ambient temperature (cold shock) on the spores' susceptibility to thermal inactivation strategies.
- Assess the inactivation strategy's ability to achieve a 3-Log reduction; 99.9% probability of kill. If achieved, determine if the data can be used to extrapolate a 6-Log kill probability.
- Further assess the use of *Bt* as a comparatively safe surrogate organism for *Ba*.

1.5 Scope of Work. The original ambitious plan for this research was to accomplish the following:

- Use applied microlithography techniques to create a platform for population with *Bacillus anthracis* and *Bacillus thuringiensis* spores. Each slide platform will be comprised of 532 etched wells with an approximate diameter of 5μm and 3-5μm in depth.
- Use established methods to culture, grow and harvest *Bacillus anthracis* and *Bacillus thuringiensis* spores. Concentrated spore slurry will be used to populate

the sample platform; vortexing, washing and centrifugation will remove majority of bio debris leaving spore concentrate.

- Use a Nd:YAG laser to indirectly heat the spores using a lab produced, custom black body radiator for various time and temperature ranges; times from 0.1 to 3 seconds and temperatures ranging from 300 to 1300K.
- Use and refine manual counting techniques to determine well population and post thermal treatment inactivation probability. Application of Malachite Green Staining protocol for greater clarity of spore population; fluorescent staining techniques. Further assess toxicity or impact of this application on spore viability.

2. Literature Review

2.1 Organism Significance

Ba is far from being the only biological agent of concern when assessing the defense of American citizens. The United States Centers for Disease Control and Prevention (CDC) defines a bioterrorism attack as a the "deliberate release of viruses, bacteria, or other germs (agents) used to cause illness or death in people, animals, or plants" (Department of Health and Human Services, 2007). The CDC divides biological agents into three categories: Category A, B, and C agents. Classification is made based on a number of factors to include the transmission, mortality rates, available treatments, and impacts on public health and order. Category A agents are considered the highest risk with Category C agents being the lowest. *Ba* is listed by the CDC as one of six Category A agents (Department of Health and Human Services, 2007).

Bacillus anthracis (Ba) is a bacterial organism that is the causative agent of the animal disease anthrax. It has been a recognized, studied organism since Robert Koch established it as the cause of disease in livestock in 1876; in essence the birth of modern microbiology. Anthrax was also the first disease for which a bacterial vaccine was developed; Louis Pasteur in 1881 (Pasteur, 1881). *Ba* does not pose the person to person transmission risk as do other biological agents like the smallpox virus. It also, does not possess the greatest toxicity; *Clostridium botulinum* toxin is the most toxic compound per weight of agent (Department of Health and Human Services, 2007). So, with this much opportunity for study, existing knowledge, a vaccine, and difficult route of transmission why is *Ba* any more feared than the common flu? *Ba* is one of, if not the, most effective

biological agents due to the resistance of its spores to a wide range of environmental conditions and decontamination methods. This same resilience makes *Ba* the hardest inactivation target for counterproliferation efforts. A spore is a metabolically dormant cell produced by the organism during periods of limited, or no, nutrients. Spores can remain viable 'for many years, certainly hundreds' in this dormant state until nutrients again become available resulting in a resumption of spore germination, outgrowth and metabolic activity (Setlow, 2005). *Bacillus* spores, in general, have shown a resistance to heat, radiation and chemical decontamination methods well beyond that of toxins, viruses, fungi and many other bacteria.

A biological agent is not a biological weapon until a practical means of dispersion and/or transmission is provided for; ie: aerosol, water transport, and/or food borne. Spores, give *Ba* an advantage over other biological agents because they can survive energetic dispersion techniques such as aerosolization. Biological agent susceptibility to environmental degradation is greatest for vegetative bacteria, intermediate for viruses, and least for bacterial spores (Stuart & Wilkening, 2005). If *Ba* spores can be destroyed by a particular method of decontamination, it is generally accepted that the method will be effective for other threat biological agents with potential for use in biological weapons (Richardt & Blum, 2008).

2.2 Endospore

2.2.1 Function

Vegetative cells are bacteria that are actively growing, metabolizing and dividing. When vegetative cells are subjected to environmental stresses such as nutrient deprivation they eventually die. Some bacteria can mitigate the problems associated with environmental stress by converting to a non-growing, or resting, state in the form of spores or cysts. These states have very low to nonexistent rates of metabolism. There are several types of spores. Some are highly resistant structures that are formed under conditions of cell stress and are created inside a supportive cell and are termed endospores. 'Endospores are a subclass of spores that are very resistant to harsh conditions' such as lack of nutrients, chemicals, high heat, radiation, and dessication; (Roberts, 2006). Other spores are part of the normal reproductive cycle, being created by differentiation of a vegetative cell and we will refer to these as spores.

2.2.2 Structure

Four distinct layers are observed and discussed in context with the endospore, they are the exosporium, spore coat, cortex and core (See Figure 1).

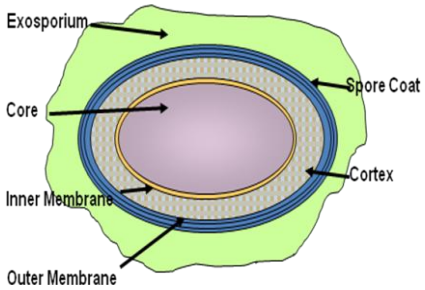


Figure 1. Bacillus Spore Structure. Features not drawn to scale. Shape and size of exposporium varies widely across the species (from Goetz, 2005)

Exosporium. *Ba* endospores characteristically have a loose-fitting outer surface called an exosporium. The exosporium is composed of a basal layer and an external 'hair-like nap' (Christopher T. Steichen, 2005). The exosporium is the primary site of contact with the environment and any host defenses such as surface antigens. It is a semipermeable barrier that excludes large, potentially harmful molecules such as antibodies and hydrolytic enzymes. The filaments of the exosporium are formed by a single collagen-like glycoprotein called BclA, whereas several different proteins form or are tightly associated with the basal layer making up a highly cross linked keratin (Phillip Gerhardt, 1961).

Spore Coat. The spore coat is composed of several protein layers that are impermeable to most chemicals and biocides. The coat is composed of more than two dozen different types of proteins and there is some evidence that these proteins are connected by cross-links. These covalent connections between coat proteins are believed to contribute to the spores' resistance (Roberts, 2006). The spore coat is further divided in to the outer and inner spore coats. The outer spore coat has been shown to be disulphide rich and which is believed to contribute to alkali-resistance. Finally, the inner spore coat consists of acidic polypeptides making it alkali-soluble.

Cortex. The cortex is composed of two layers of loosely cross-linked peptidoglycan. The first is a thin, dense layer comparable to the vegetative cell wall. The second, is a thicker less dense layer containing modified peptidoglycan. Modifications include less cross-linking with only 3% of the muramic acid, (40% in vegetative cell wall) present allowing for an easier outgrowth during germination

(Roberts, 2006). Second, much of the muramic acid is modified to a muramic-β-lactam structure which serves as a specific target for lytic enzymes that are activated during germination.

Core. At the center of an endospore is the core. The core consists of a core wall, cytoplasmic membrane, permeable outer membrane, impermeable inner membrane (excluding hydrophilic molecules), cytoplasm and the nucleoid. The cytoplasm contains ribosomes, enzymes and everything that is needed to function once returned to the vegetative state. The nucleoid contains the DNA. The core is dehydrated, which is essential for heat resistance, long-term dormancy and full chemical resistance. Calcium dipicolinate is a major component of the core and has been shown to play a role in resistance to wet heat and UV light (Roberts, 2006).

2.2.3 Sporulation

Sporulation is a unique developmental cycle and process of differentiation that provides the bacteria with a mechanism for survival (American Society for Microbiology, 2006). Unlike the process of binary fission, endospore formation is not a reproductive process.

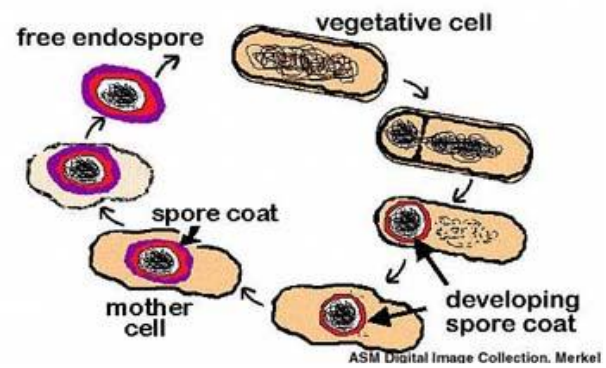


Figure 2. Sporulation Cycle (from American Society for Microbiology, 2006)

Once environmental conditions trigger the sporulation cycle, the first step is an unequal cell division resulting in the formation of a forespore septum (See Figure 2). Next, the plasma membrane of the cell surrounds this wall and pinches off to leave a double membrane around the DNA, and the developing structure is now known as a forespore. Calcium dipicolinate is incorporated into the forespore during this time. Next the peptidoglycan cortex forms between the two layers followed by a spore coat and exosporium resulting in a mature endospore. Mature endospores are spherical or elliptical. They contain a complete set of the genetic material (DNA) from the vegetative cell, ribosomes and specialized enzymes (Todar, 2009). The sporulation cycle is completed when lytic enzymes destroy the vegetative cell's sporangium resulting in the release of the endospore. Mature endospores are released from the vegetative cell to become free endospores. When the free endospores are placed in an environment that supports growth, the endospores will revert back to a vegetative cell in a process called germination. The free endospores must have access to nutrients and be exposed to elevated temperatures which will then initiate germination. (Setlow, Resistance of bacterial spores, 2000)

Table 1. Differences between endospores and the vegetative cells that form them; (from Todar, 2009).

Property	**Vegetative cells**	**Endospores**
Surface coats	Typical Gram-positive murein cell wall polymer; crystalline S-layer	Thick spore coat, cortex, and unique peptidoglycan core wall; no S-layer
Microscopic appearance	Nonrefractile	Refractile
Calcium dipicolinic acid	Absent	Present in core
Cytoplasmic water activity	High	Very low
Enzymatic activity	Present	Absent
Macromolecular synthesis	Present	Absent
Heat resistance	Low	High
Resistance to chemicals and acids	Low	High
Radiation resistance	Low	High
Sensitivity to lysozyme	Some sensitive; some resistant	Resistant
Sensitivity to dyes and staining	Sensitive	Resistant

2.3 Agent Neutralization Strategies

Decontamination is defined as the "irreversible inactivation of infectious agents so that an area is rendered safe" (Anderson, Novak, Keith, & Elliott, 2008). Sterilization is "the complete destruction or elimination of microbial viability; including spores" (Anderson, Novak, Keith, & Elliott, 2008). For the purposes of this discussion, decontamination and sterilization techniques will be generally referred to as inactivation strategies. The body of work does not definitively conclude what defines "complete destruction of" or when an area is "rendered safe". The aforementioned definitions do not speak in terms of spores, colony forming units, infections or casualties. To achieve an infectious dose to a human, roughly 8,000-50,000 *Ba* aerosolized spores are required (US Army Blue Book, 2005). Much of the empirical work displays data in terms of log reductions versus infective dose quantities. For example, a sample of *Ba* spores when treated with boiling water for 10 minutes will show a "reduction in spore counts by at least 10^6, or a 6-Log reduction in spores" (Whitney, et al., 2003). This sterilization process displays a spore reduction capability of six orders of magnitude which means the survival probability of the original number of spores is now 10^{-6}. Another way to understand this terminology is to consider each Log reduction a 90%, or one decimal place, decrease in the original number of spores (U.S. Food and Drug Administration, 2007). In the boiling water example above, the probability that all of the spores from the original sample were inactivated is 99.9999%.

Several inactivation strategies using radiation, wet heat, and chemical reactions have been studied, but have limited empirical data with the gaps being filled through

mathematical modeling. Inactivation strategies have seen a large growth in commercial production and have included methods based on heat, radiation, formaldehyde, hypochlorite solution, chlorine dioxide applications and combinations of the same. For many of the commercial off the shelf products and strategies there are concerns relevant to the consistency in methodology, environmental conditions, organisms and many other variables during testing and fielding. One such criticism is the use of low risk surrogates in laboratory settings, such as *Bacillus thuringiensis* (*Bt*), and other members of the *Bacillus* genus to replicate more virulent biological agents such as *Ba*. Additionally, various surfaces and animal toxicokinetic models have been used to determine the effectiveness of these strategies, but they are not all inclusive and may not accurately reflect human response.

2.4 Thermal Inactivation of Endospores

Endospores can withstand brief exposure to very high temperatures. Microorganisms and spores are more tolerant of dry heat. The term dry heat does not imply that no water is present, but the water is not supplied from an external source. In treatment strategies using moist heat, the organisms' surrounding environment has a relative humidity of 100%. Microorganisms are being heated directly in water or a water-saturated atmosphere. Dry heat, therefore, includes any conditions in which the relative humidity is less than 100% (Block, 1991). For long exposure times moist heat is more lethal. Generally, endospores survive 10^3 times longer in dry heat than wet heat (K. Fox, 1969). For wet heat, the primary damage is to the spores' protein and DNA. For dry heat, damage to the spores' DNA dominates with single strand breaks in particular

being caused by depurination. Generally, threshold thermal damage accumulated in spores is largely due to hydrolysis-type reactions of DNA in the spore core.

2.4.1 Fundamental Chemical Reactions

Maillard Reaction. The Maillard reaction produces reactive water in the spore at high temperatures which can damage DNA and enzymes through hydrolysis reactions. The Maillard reaction is 'a chemical reaction between an amino acid and a reducing sugar, usually requiring the addition of heat' (Chang, 1994). It is a form of non-enzymatic browning where a reactive carbonyl group of a sugar interacts with the nucleophilic amino group of an amino acid (see Figure 3). The interacting proteins and sugars produce water, (a condensation reaction), which ultimately degrades reactants to organic acids. The initial dehydration step is reversible, (through hydrolysis), but succeeding steps cannot be reversed. The reaction occurs less readily in the presence of water.

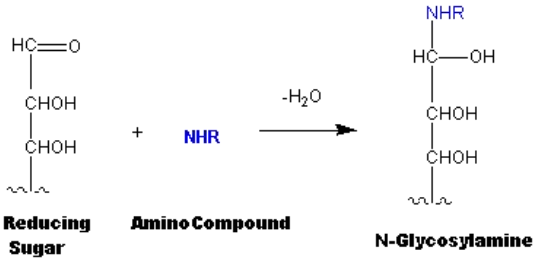


Figure 3. Maillard Reaction (from Burggraf unpublished)

Hydroylsis. Hydrolysis is 'a chemical process in which a molecule is cleaved into two parts by the addition of a water molecule' (Mandel & Rowley, 1963). Hydrolysis reactions occur spontaneously upon exposure to water. Water can be made available when it is desorbed from materials or when it is freed from molecular bonds

during heating by reactions such as the Maillard reaction, as is observed in dry heating. Also, water for hydrolysis can be made available from the surrounding environment, as seen wet heating. Some organic molecules, like DNA and catalytic proteins, are more fragile than ordinary hydrocarbons. They can be damaged by reactions having lower activation energies such as reaction with water or hydrolysis reactions. In example, a common method for sterilization of equipment and waste is an autoclave. Materials are commonly sterilized at 121°C for 20 minutes under a pressure of at 15psi, hot water vapor. Because the water activity generated is so large, this time/temperature sterilization condition is comparatively low in temperature and long in duration compared to dry heating sterilization conditions. In the autoclave, microorganisms are inactivated in a high moisture environment at temperatures above the boiling point of water, but very much lower water activity than that which is achieved during dry heating. So sterilization by dry heat requires higher temperatures and longer heating times.

Pyrolysis. Pyrolysis is the 'as chemical decomposition of organic materials by heating in the absence of oxygen' (Environmental Protection Agency, 1992). Comparatively, pyrolysis reactions will occur at significantly higher temperatures than the Maillard and hydrolysis reactions described above, because more energy is required to activate pyrolysis reactions. Pyrolysis reactions occur when organic materials are rapidly heated to 450-600°C in absence of air (Shah, Schultz, & Daiga, 1989). Under these conditions organics are transformed into gases, small quantities of liquid, and a solid residue containing carbon. As rapid heating occurs, violent vibration and subsequent breakage at random positions throughout the molecular structure.

Hydrocarbon molecules, including all chemical agents and biological molecules, are converted to harmless compounds by pyrolysis at temperatures above 600° C. Arrhenius equation gives the dependence of the rate constant (k) of chemical reactions on the temperature (T) (Arrhenius, 1903).

Equation 1. Arrhenius equation with constants

$$k = Ae^{-\frac{E_A}{RT}}$$

A=frequency factor ; constant at low temperatures [sec^{-1}]
e = mathmatical quantity; e=2.71828
R= gas constant; R=8.314472[$JK^{-1}mol^{-1}$]
T= temperature [K]
E_A = activation energy [kJ/mol]

Consider a pyrolysis example which can be extrapolated to other hydrocarbons, assuming that all C-C bonds take about the same amount of energy to break, regardless of the molecule they are in. For the purpose of this discussion we will accept the findings of Alami et al that determined the activation energy (E_A) required for the pyrolysis of a propane molecule (C_3H_8) is equal to 86.4 kcal /mol (361.50 kJ/mol) (Al-Alami & Kiefer, 1983). Note that the average bond enthalpy of a C-C bond is 348 kJ/mol and that these reactions generate radicals, (ie: methyl group):

$$C_3H_8 \rightarrow CH_3 + C_2H_5$$

These radicals can propagate the chain pyrolysis process by abstraction reactions in which the hydrogen is removed from another molecule producing another radical. Using a frequency factor of $Log_{10}A=17.17$ [cm^3, s, mol] apply Equation 1 to find k. Using the

value for k, determine how long the average hydrocarbon bond will last by assuming a first order decomposition model shown by Equation 2.

Equation 2. Half-life equation using Arrhenius rate constant for k.

$$t_{\frac{1}{2}} = \ln \frac{2}{k}$$

k = Arrhenius Rate Constant

Table 2 below shows the half life of a propane molecule exposed to 600°C is equal to 13.74sec^{-1}. For biological molecules, pyrolysis easily achieves threshold inactivation damage given temperatures greater than 600°C for durations on the order of a second. As the temperature decreases, the half life of the molecule increases exponentially. However, pyrolysis is shown to be a less effective hydrocarbon degradation mechanism at lower temperatures where hydrolysis reactions are important. Hydrolysis reactions have lower activation energies than pyrolysis reactions.

Table 2. Results of Arrhenius Calculations using empirical rates for the pyrolysis of propane.

A	[sec-1]	9.22E+15	9.22E+15	9.22E+15	9.22E+15
E_A	[kJ/mol]	361.5	361.5	361.5	361.5
T	[K]	873	673	473	273
k	[sec^{-1}]	2.16E-06	8.06E-13	1.10E-24	6.23E-54
$t_{1/2}$	[sec^{-1}]	13.738902	28.540172	55.858371	123.20324

2.4.2 Damage Mechanisms

During thermal treatment, the spore system is subjected to a competition of pyrolysis and hydrolysis reactions. The threshold inactivation is likely achieved by the combination, and cumulative effects, of both reactions. Which reaction predominates depends on the temperature and moisture content, both from free water and water freed

by chemical reactions like the Maillard reaction. There are two types of water pertinent to a spore system and they are free water and molecularly freed water. The wet heat system will have sufficient water available for the hydrolysis reactions to begin at lower temperatures, likely resulting in hydrolysis being the dominant reaction. In an ideally dry system, hydrolysis will not occur until molecular water has been freed by the supporting Maillard-type reaction. If sufficient water is not liberated, or present, then as temperatures continue to rise the dominate reaction will be pyrolysis.

Dry Heat. Extreme heat causes portions of the double helix DNA molecule to denature and cleave apart. DNA is the critical component for organism replication, however, it is chemically reactive. DNA undergoes spontaneous decomposition in vegetative cells by hydrolysis, oxidation and non-enzymatic methylation of DNA. Vegetative cells have sophisticated biochemical machinery to recognize and repair this DNA damage. These repair mechanisms are inactive in the spore, so any DNA damage accumulates.

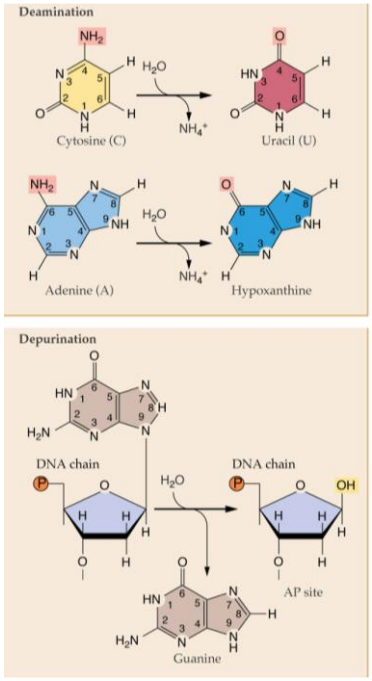


Figure 4. Schematic of Deamination and Depurination (from Setlow, 2000)

Two hydrolysis reactions are the dominant degradation mechanisms for DNA in a dry thermal environment. These reactions are deamination and depurination, (see Figure 4). Deamination is the loss of a pyrimidine base, (nucleotides cytosine or uracil), from a DNA molecule when spontaneous hydrolysis of cytosine changes it to a uracil. Cytosine base bonds are the most

susceptible to deamination. The second, and more prominent, of the two mechanisms is depurination which is the loss of a purine base, (nucleotides adenine or guanine), from the deoxyribose-phosphate backbone of the DNA molecule (Barbara Setlow, 1995). Guanine sites are slightly more reactive than adenine. Depurination occurs spontaneously in presence of water and is accelerated at low pH and high temperatures.

Molecularly bound water becomes mobile on the order of milliseconds at high temperatures (Baker & Burggraf, 2008) enabling it to move into the spore core. There, available water can react by hydrolysis reactions to degrade the DNA. Temperature at which this occurs varies depending not only upon the water activity, but upon nucleotide targeted and the activation energies for hydrolysis at the respective chemical bonds (Ana Neacsu, 2008). It has been shown that purine bases are released from DNA at neutral pH and temperatures near the helix coil transitions temperature between 45 to 80° C (Mandel & Rowley, 1963). The wide range of temperatures may be caused by the presence of degradation products from DNA shearing and ribonucleic acid damage (Mandel & Rowley, 1963). Depurination of DNA has been empirically shown to occur at a significant rate; with '…a rate constant $4x10^{-9}sec^{-1}$ at 70°C and a pH of 7.4. The activation energy of the reaction is 31±2kcal/mole', (Lindahl & Nyberg, 1972). DNA deamination studies show activation rates for deamination of cytosine residues at $9.5x10^{-9}sec^{-1}$ at 70°C and a pH of 7.4. The activation energy of the reaction is in the range of 26-28 kcal/mol (Lindahl & Nyberg, 1972). The N-glycosidic bonds between a purine base and its deoxyribose chain are the most susceptible to hydrolysis. This susceptibility to depurination hydrolysis is probably the cause of the relationship between core water

content and thermal inactivation by dry heating for long times (Greer & Zamenhof, 1962).

Wet Heat. For wet heat inactivation of endospores the primary target is the protein followed by the DNA. Proteins are long strands of amino acids linked together in specific sequences; as dictated by genetic coding. The lack of cell mutations associated with wet heat, compared to dry, indicates that protein hydrolysis damage dominates in wet heating. Regardless of whether the DNA blueprints remain intact, if there is no protein to serve as the brick and mortar for repair, then no attempt can be made—let alone a faulty one driven by damaged DNA. Each protein molecule within a spore has a specific function, from structural supports to individual catalysts for every biochemical reaction. It is difficult to establish what protein, within a dormant endospore, must be damaged to achieve an inactivation. It has been suggested that the protein responsible for critical germination functions is responsible for the decisive damage.

Studies have shown that certain inactivation strategies, such as heat, impact germination essential enzymes differently including; specifically glucosaminidase and lytic transglycosylase.

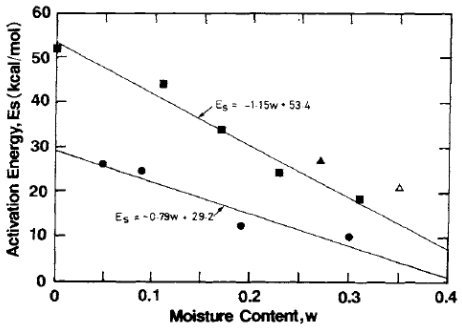


Figure 5. Dependence of the activation energy of soy-protein denaturation (from Yoshi et al)

These lytic enzymes were shown to be impacted by a thermal treatment of 90°C for 15minutes. Analysis showed that in heat treated *Bacillus subtilis*, 'revealed a reduction of >85% in glucosaminidase and epimerase products, when compared to untreated spores' (Atrih & Foster, 2001). Additionally, spores thermally treated during the study showed an 'impaired cortex hydrolysis/modification, but with <20% loss in viability' (Atrih & Foster, 2001). DNA damage must be repaired by active proteins before germination can be completed. Hawkins showed for *Ba* spores damaged by dry heat that the germination time increased with increasing temperature; approaching the temperature of complete inactivation. The longer times for germination are due to the more significant DNA damage that must be repaired or to the less capable repair proteins. Perhaps a combination of both.

Protein denaturation is strongly influenced by water content. Work has shown that the apparent activation energy for protein denaturation increases as moisture content decreases; as shown in Figure 5 (H.Yoshii, Furuta, & Noma, 1990). Yoshi et al found that 'the log of the apparent first order rate constant for solid protein denaturation increases linearly with apparent activation energy, as expected by Arrhenius relationship, but with a very small frequency factor' (H.Yoshii, Furuta, & Noma, 1990). The model does not account for the different sources of water feeding the hydrolysis reactions; environmental versus freed molecular. A bimolecular hydrolysis reaction, having low activation energy, contributes more damage in a high moisture environment and less in that of a low.

2.4.3 Resistance Mechanisms

Three resistance mechanisms have been recognized in the thermal susceptibility of *Bacillus* spores: water content, small acid soluable proteins, and dipicolinate salts. As indicated above, the presence of available/freed water in a spore system contributes to the degradation of DNA. 'Reduced core water (lowers) the amount of water associated with spore proteins, thus stabilizing them to thermal denaturation' (Nicholson, Munakata, Horneck, Melosh, & Setlow, 2000). In growing bacterium, 75-80% of the wet weight is water….in the spore core itself only 25-35% of the wet weight is water (Gerhardt & Marquis, 1989). Calcium content increases resistance to wet heat and oxidation. The higher the level of core divalent mineral ions, the more wet-heat resistant are the spores. This effect appears to be due in part to a decrease in core water with increasing core mineralization (Gerhardt & Marquis, 1989).

Another factor in *Bacillus* spore resistance to UV, thermal and oxidizing inactivation strategies is a group of DNA binding proteins termed small, acid-soluble proteins (SASP) of the α/β type. Saturation of spore DNA with SASP greatly slows DNA depurination caused by heat as well as hydroxyl radical-induced DNA backbone cleavage (Setlow, 1995).

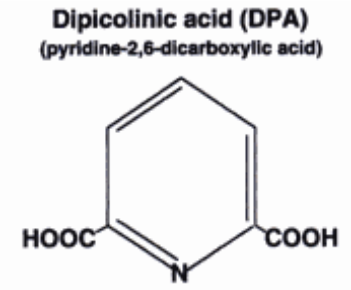


Figure 6. Chemical structure of Dipicolinic acid (DPA), (from Setlow 2000).

Finally, DNA is immobilized in an inert matrix of dipicolinate salts, mostly calcium dipicolinate (CaDPA), which constitutes up to 10% of a spore's dry weight. The role of solid CaDPA in lending heat resistance to spores has been attributed to immobilization of essential enzymes and nucleic acids (Setlow, Resistance of bacterial spores, 2000). Because of the immobilization of DNA in CaDPA, repair cannot occur in the spore state. Successful repair of the double stranded DNA structure must be delayed until germination when cell metabolic activities continue and sufficient water is available to rehydrate. Repair during germination is critical to the reconstitution of a vegetative cell. DNA repair by enzymes early in germination confers resistance to alkylating agents, dry heat, genotoxic chemicals, and UV radiation.

Finally, a strong, highly cross-linked keratin spore coat contributes to endospore resistance to chemicals and sterilization agents. The spore coat is impermeable to hydrophilic chemicals greater than 200 atomic mass units (Gerhardt & Marquis, 1989).

2.5 Thermal Diffusion Model

Previous attempts to measure the inactivation probability for *Ba* spores at short times and high temperatures have suffered because of the times necessary to achieve a steady state temperature for large samples. Modeling was done to determine the most important design parameters. The following model shows the relationship between the source temperature and the temperature sample spores are exposed to during thermal treatment using the experimental sample configuration shown in Figure 7. Early analysis of the heating methodology led to questions regarding the ability of the work to characterize exactly what temperature the spore is being exposed to. The temperature

emitted at the surface of the black body irradiator (T_s) can be determined from the spectra collected during thermal treatments. The steady-state temperature at the surface of the black body is different than the temperature seen by the spore (T_o) due to heat conduction across an air gap between 0-25µm. The steady state model takes in to account the sample configuration to include the two prevalent mediums; glass and air (Baker unpublished, 2009). The predicted spore temperature relative to the temperature of the black body radiator is shown in Figure 7. In this work carbon black is used to approximate a black body radiator.

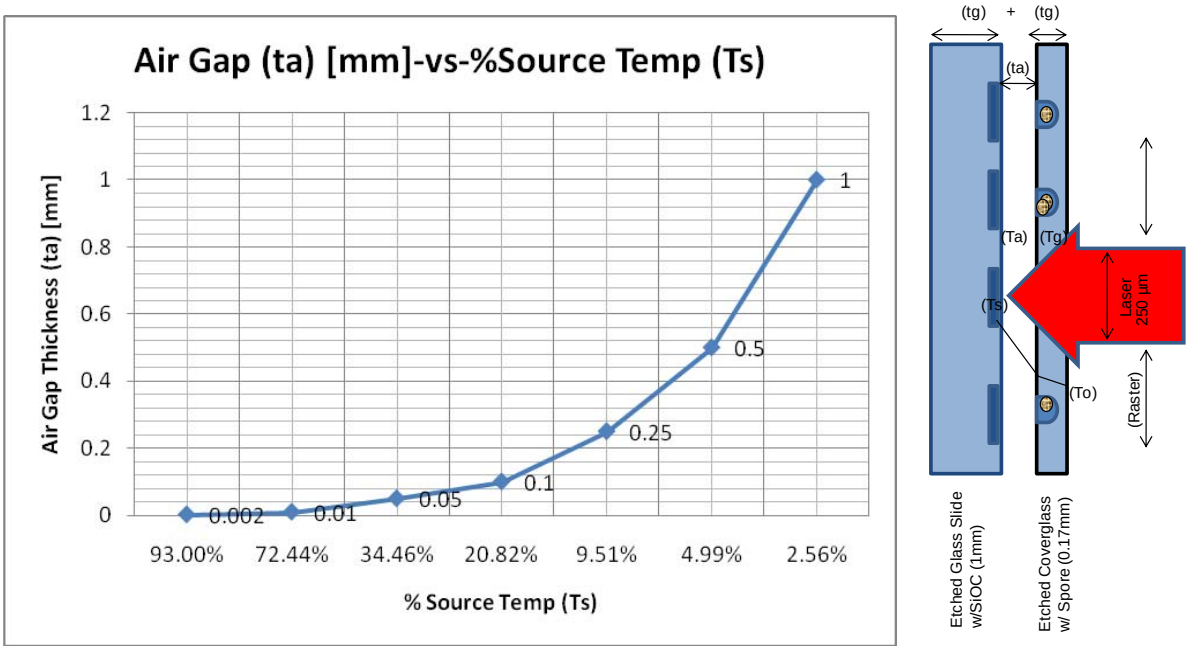


Figure 7. Percentage of the source temperature observed by the spores during thermal treatment with corresponding diagram of temperature loss from source temper (T_s) to temperature observed by the spore (T_o)

Terms Defined:

K_a =Thermal Conductivity of Air
K_g =Thermal Conductivity Soda Lime (Glass)

T_s =Source Temperature
T_g =Glass Temperature
T_o =Ambient Air Temperature (air on laser side of glass)
T_a =Air Temperature (air gap between irradiator and spore)

t_a =Thickness of Air Region (air gap between irradiator and spore)
t_g =Thickness of Glass (complete sample configuration)

Derivation and Conditions:

$T_a(x) = T_s - ax$ $(0 < x < t_a)$
$T_g(x) = T_o + b(t_a + t_g - x)$ $(t_a < x < t_g)$
Note: a & b determined by satisfying continuity conditions at $x=t_a$

(1) $T_a(t_a) = T_g(t_a)$

(2) $K_a \frac{\delta T_o}{\delta x}(t_a) = K_g \frac{\delta T_g}{\delta x}(t_a)$

Note: Continuity flux condition; flow at boundary

From these conditions...

$T_s - at_a = T_o + bt_g$

$-K_a a = -K_g b$

$$\Rightarrow a=(T_s - T_o)\frac{K_g}{K_g t_a + K_a t_g} \qquad b=(T_s - T_o)\frac{K_a}{K_g t_a + K_a t_g}$$

Let $\Delta T = T_s - T_o$ & $\mu = \frac{K_g t_a}{K_a t_g}$

Then $a=\frac{\Delta T}{t_a} \bullet \frac{\mu}{1+\mu}$ $\qquad b = \frac{\Delta T}{t_g} \bullet \frac{1}{1+\mu}$

Now $T_a(x) = T_s - \frac{\Delta T}{t_a} \bullet \frac{\mu}{1+\mu} x$

$$T_g(t_a) = T_o + \frac{\Delta T}{t_g} \bullet \frac{1}{1+\mu} \bullet x(t_a + t_g - x)$$

$$T_g(t_a) = T_o + \frac{\Delta T}{t_g} \bullet \frac{1}{1+\mu} \bullet t_a(t_g)$$

$$T_g(t_a) = T_o + \frac{t_a}{1+\mu} \bullet \Delta T$$

2.6 Sol-gel Process

The sol-gel process is roughly described as the chemical production of solid materials starting from liquids. The sol-gel process produces an inorganic network by forming a colloidal suspension (sol) and gelation of the sol to form a network in a continuous liquid phase (gel) (Brinker & Scherer, 1990). For the purposes of this work, the sol-gel process was used to apply a uniform, protective siloxane coating atop of the carbon black body irradiators as an oxygen diffusion barrier. Carbon black closely approximates a black body radiator at low temperatures. However, near 800^{o}C it can rapidly oxidize like charcoal, limiting its utility. In essence, the sol-gel process converts an inorganic gel to glass at a relatively low temperature; compared to temperatures normally associated with glass production. Function of this layer was to stabilize carbon as well as expel any trapped air interspersed between carbon black particles. A sol is a dispersion of the solid particles (~ 0.1-1 mm) in a liquid where only the Brownian motions suspend the particles. A gel is a state where both liquid and solid are dispersed in each other, which presents a solid network containing liquid components (Lev, et al., 1995). Three reactions are generally associated with the sol-gel process: hydrolysis, alcohol condensation, and water condensation.

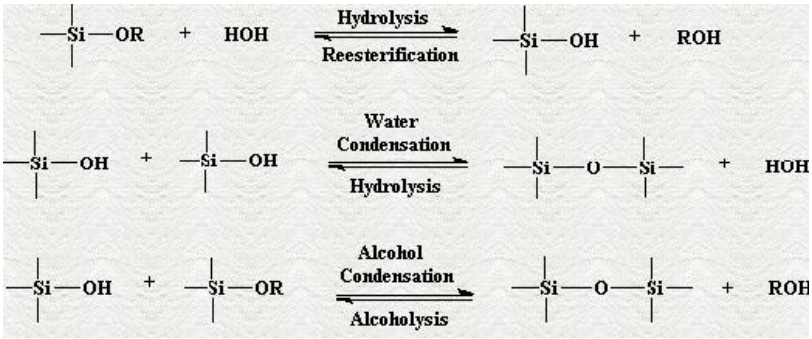


Figure 8. Instrumental reactions to the sol-gel process (from Mauritz, 2004)

The rate of the hydrolysis and condensation reactions is impacted by factors such as pH, nature and concentration of catalyst, H_2O/Si molar ratio, and temperature. The properties and structure of the inorganic network produced can be varied by controlling these factors. The hydrolysis reaction replaces alkoxide groups (OR) with hydroxyl groups (OH) through the addition of water. The subsequent condensation reactions produce siloxane bonds (Si-O-Si) plus the by-products water or alcohol from the silanol groups (Si-OH). Acid or base catalysts are used to ensure a rapid and complete hydrolysis. Under acidic conditions, an alkoxide group may be protonated in a rapid first step. Electron density is withdrawn from the silicon atom, making it more electrophilic and thus more susceptible to attack from water (Brinker & Scherer, 1990).

3. Materials and Methods

3.1 Materials and Equipment

3.1.1 Biological Agent and Simulant

The bacteria used for this study was *Bacillus anthracis*, Sterne strain *(Ba (Sterne)*. *Ba (Sterne)* is a nonencapsulated toxigenic strain commonly used in livestock vaccination. *Ba (Sterne)* produces sublethal amounts of the toxin which then initiates formation of protective antibodies in the animal's immune response (Todar, 2009). The samples of *Ba (Sterne)* used in this research were obtained from Dr. Eric Holwitt of the Air Force Research Laboratories, Biomechanisms and Modeling Branch, Brooks City Base, Texas.

Bacillus thuringiensis subspecies *kurstaki, (Bt (kurstaki)).* Two stock quantities of *Bt* were provided by Ms. Yvette B. Gonzalez, the Program Manager/Primary Investigator, of the BioAgent Defeat Assessment Programs Air Force Research Laboratories out of Brooks Air Force Base, Texas. The first stock, referred to hereafter as *Bt* (Javelin), is a commercially produced biological insecticide obtained from Certis© L.L.C., out of Columbia, Maryland. *Bt* (Javelin)'s is specifically used against the larvae of insects belonging to the lepidopterous family such as the Fruitworm, Cotton bollworm, and Gypsy moth. Composition of the product by percentage weight is "85% *Bacillus thuringiensis*, subspecies *kurstaki* solids, spores and Lepidopteran active toxins…15% Other Ingredients", (Certis U.S.A. L.L.C.). The two other ingredients listed on the product's material safety data sheet (MSDS) are a surfactant and Sodium Sulfate though

the exact quantities are not represented. *Bt* (Javelin) is the darker of the two stocks, is rusty brown in color, granular and has fish meal odor. The second stock, referred to hereafter as *Bt* (Toast), is comparable but underwent additional processing at Dugway Proving Grounds. *Bt* (Toast) is lighter in color than *Bt* (Javelin) due to added flow enhancers; such as silicone. All samples were stored in the form of lyophilized spores and kept in glass culture tubes at room temperature. All samples were handled and stored in accordance with safety and security protocols outlined in the U.S. Department of Health and Human Services, 'Biosafety in Microbiological and Biomedical Laboratories', 5th edition published in 2007. *Ba (Sterne)* strain organisms were handled in accordance with Biosafety Level 2 guidelines. Sterilization of all equipment and materials directly contacting biological samples is accomplished using a Tuttnauer®/Brinkmann™ 3870 autoclave. Chemical sterilization can be accomplished using household bleach (5.25-6% sodium hypochlorite) with a contact time of 10 minutes. Due to the corrosive properties of bleach, sterilization of metal work surfaces or equipment can be accomplished using methanol; less effective. Contamination prevention is the best means of maintaining a sterile work environment.

3.1.2 Carbon Black

Planck's Law states that the thermal radiation emitted by a blackbody, heated to a given temperature, emits thermal radiation with the same spectrum (Eisberg, 1985). A uniform deposition of carbon black, which has strong absorbance from the ultraviolet to the infrared, nearly performs as a perfect black body. This deposit is capable of

absorbing all light that strikes it and radiating energy back out to its surrounding environment. Carbon black is near pure elemental carbon produced through the incomplete combustion or thermal decomposition of hydrocarbons; gaseous or liquid. At the macroscopic level its physical appearance is that of a black, finely divided pellet or powder. At the microscopic level its form is that of a spherical or grape-like (aciniform) particulate. Its particle sizes are in the range between 0.01-0.3µm in diameter, but due to London attractive forces particulates are generally found as part of much larger conglomerates between 0.1-1µm (Ann Watson, 2001). London dispersion forces, sometimes called Van der Waals forces, are weak intermolecular forces that arise from the attractive force between transient dipoles in otherwise nonpolar molecules (French, 2000). Carbon black's traditional use is as a reinforcing agent in tires. Because of its specific surface area, particle size, structure, conductivity and color the uses of carbon black have expanded. Common applications include pigmentation, ultraviolet (UV) stabilization and conductive agents in rubber/ plastic products, printing inks and coatings (Ann Watson, 2001). It's these same unique properties described above that make carbon black ideal for use in the production of a micro-black body irradiator used in heating the biological samples of interest. The carbon black used during this study was obtained from Fisher Scientific®, 'Carbon Lampblack (C198-500).

3.1.3 Photomask

Organizational masks on hand that were used in prior research (Hawkins, 2008) utilized well sizes of 5 and 10µm respectively. The well size, pattern and spacing were less than ideal for use in producing custom biological and carbon black radiator; scaled at

a 1:10 ratio respectively. Additionally, the previous mask and exposure protocol did not facilitate the mass production of identical etched platforms. Two new photomasks were procured from Photo Sciences, Inc© out of Torrance, California. The photomasks are custom made based on digital designs provided by the customer or created by Photo Sciences, Inc©. The preferred data format is Graphic Data System II (GDSII) followed by AutoCAD® Drawing Interchange Format (AutoCAD DXF). Photomasks used in this study were constructed from chrome/soda lime (material/substrate) and are 4x4x0.060" in size. Tolerances and uniformity features are certified to ±0.25 µm. Wells are 5 and 50µm respectively with a spacing of 1.5mm between rows/columns and from the edges. There are 532 wells per mask template; 38 columns by 14 rows. The aforementioned well sizes were selected based on previous work using 5µm for the biological sample preparation. The 50µm well size was selected based on the size of the biological well; ten times in size to facilitate carbon/biological alignment during final preparation of the sample. The 1.5mm spacing was based off of the early hypothesis regarding the thermal propagation through the samples' micro-etched glass platforms. Specific concern was the premature, or redundant, heating of neighboring wells. Finally, 'L-shaped' alignment brackets were included at the perimeter corners to help with macro and microscopic alignment; 3mm is an average size used for observation with the naked eye. Final configuration has three identical row/column templates contained within alignment brackets at the four corners. This design was intended to be used for purposes of batching with an increase in output, repeatability and quality control. Intention was the production of three identical platforms per exposure versus just one; see paragraph '3.2.1

Etched Platform Production'. Diagrams showing the photomasks' final dimensions and layouts are shown in Appendix B.

3.1.4 EVG620 Mask Aligner

Any light source with near-UV emissions can be used to expose the photoresist to create microlithographic images. The EV Group's© EVG620 precision mask and bond aligner system was used for exposure of photoresist on glass slides, see Figure 8. The EVG620 is mostly automated and affords the researcher repeatable exposures, reduced exposure time, and improved precision compared to older systems such as the Karl SUSS MEMs mask aligner. The mask and samples can be aligned exactly each time using platform guides and controls machined to a high precision.

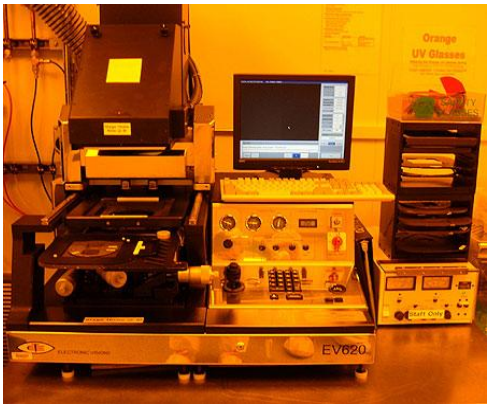


Figure 9. EVG620 Mask Aligner

Thickness and processing variable can affect exposure. Exposure times of 1-10 seconds are typically sufficient, providing the light source yields a light intensity of at least 100 mW/cm^2; at the substrate surface. In accordance with the EVG620 Product guide, it features a mercury arc lamp, variable at 350/500/1000W, and operational wavelength,

200-240/240-280/280-350/350-450nm. In its current configuration the EVG620 irradiates at 500 W/cm2 and a wavelength of 350-450nm.

3.2 Experimental Procedure

The purpose of this chapter is to highlight experimental apparatus and protocols used in this study. The experimental protocol was developed to determine the probability of kill for a thermal inactivation strategy for use against *Bacillus anthracis (Ba).* Target heating durations and temperatures are comparable to that of an explosion generated by conventional munitions; 300-1600°K for times of 0.01 to 3 seconds. Applied microlithography techniques were used to produce biological and carbon black body irradiators from etched glass. Techniques to populate the sample wells with a concentrated spore sample were explored. Endospores are refractile, light cannot penetrate them, so that they are very easy to see and detect in the phase microscope. For this same reason, they cannot be directly heated by the laser. The spores are heated using a black body irradiator. Spores are heated against black carbon wells using a solid state laser (Nd: YAG). Variations in heating temperatures and durations will be varied using adjustable raster rates and laser powers. Temperature measurements will be made using a Fourier-Transform Infrared (FTIR) spectrometer with a sensitive Mercury Cadmium Telluride (MCT) detector, from which the temperature was obtained by fitting a Planck function to the infrared spectrum from carbon. Samples were characterized before and after germination using optical microscopy and manual counting techniques. The statistical correlation between heating time, heating temperature, and spore viability was analyzed.

The methodology listed below is based on what was determined to be the final protocol for each activity. Failed techniques and trials are highlighted under several of the appendices. Though, instrumental in the learning process, their inclusion in the immediate text below would detract from the protocol.

3.2.1 Etched Platform Production

The process of creating etched platforms from microscope slides and coverglass has been explored in previous work, (Hawkins, 2008). The methodology listed below is a modification of the techniques outlined in that work and in standard operating procedures used in the Air Force Institute of Technology's (AFIT) course, "EENG 717 Standard Photolithography and Pre-Metallization" (Starman, 2008). Etched platform production can be accomplished using the following five steps: Preparation/Photoresist, Exposure, Development, Etching, and Cleaning. Source platforms for this process are Corning© Micro Slides (3"x1"x1.06mm) and Gold Seal® Cover Glass, #1 (22 x 60 x 0.17mm).

- Preparation/Photoresist
    - Mark each platform using a diamond tipped engraving pen. Recommended marking pattern is to engrave a 'B' in the bottom right corner with a sequential alphanumeric in the top right. Theses minor etches can cause fractures throughout the process so caution should be taken when performing this step not etch too deeply; especially when marking the ECS. Accountability of the platforms' working surfaces, sides to be etched, must be maintained throughout the process.

- Clean each platform via the spin coater with sequential washes of acetone (CH_3COCH_3) and methyl alcohol/methanol (CH_3OH); spin for 30 seconds at 2,000rpm. Both sides of the platform should be cleaned with the working side (side not marked with 'B') being cleaned last.
- Dry with N_2 gas. Remove from spin coater and soft bake using a hot plate at 110°C for two minutes. Allow to cool.

- Exposure. Intent of exposure is to damage the applied photoresist. The damage occurs based on the configuration and dimensions of the photomask's design. The platform, or substrate, and the mask are placed in to direct contact for this exposure to reduce diffraction variance. The damage occurs when UV light, at 500 W/cm^2 and a wavelength of 350-450nm, is radiated from above the mask and platform. The UV light is then reflected by the chrome coating of the photomask, however, in areas where there is no chrome the light penetrates to the platform and damages the photoresist.

Table 3. EVG620 Settings

Mask Holder Size	4	in
Substrate Size	3	in
Separation	55	um
Thickness of Mask	3.2	mm
Thickness of Substrate(Slide)	1.1	mm
Thickness of Substrate(Coverglass)	0.2	mm
Thickness Resist	2.5	um
Exposure Time	4	sec

- Follow startup procedures in EVG620 Product Manual (EVG620 Semi-automated NIL System , 2008). Microsoft Windows© operating system is mostly self explanatory. Once initialization has completed, the program setup will display. 'Recipe 1' is default program which must then be changed to reflect the settings in Table 3. Click 'Run'.
- Once setup is complete the program will prompt the following actions: mask placement, substrate placement, run, removal and continue/exit. Mask placement in holder is with the chrome side down; long axis of template flipping from horizontal to vertical with the swinging of the sample tray into position.
- Platform placement can be repeated with good precision using only manual means; no adjustment of sample platform is necessary or microscopic alignment. Figure 10 shows the use of the platform guides and the edges of the slides/coverglass to ensure a uniform exposure of successive samples.

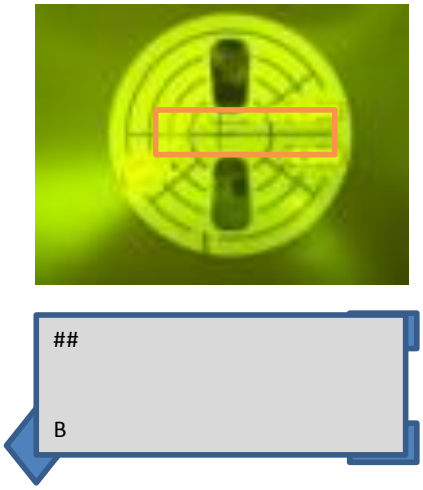


Figure 10. Placement of platform atop the EVG620 sample holder. Graphical depiction is not to scale and alphanumeric designators will be superimposed during actual emplacement.

- Development. The development step consists of the removal of the photo resist in areas of the sample that were damaged by the ultraviolet light exposure.
    - Batch platforms in production runs of five platforms. Two plastic microscope slide holders were used to hold the top and bottom of the platforms in place. This technique is especially necessary when developing/rinsing coverglass which tends to be more susceptible to hydraulic resistance. Batching of platforms is encouraged throughout the remainder of the process to help reduce variability caused by steps requiring chemical processes. Etch and development rates change over production runs based on age and exposure of chemicals to ambient lab conditions.
    - Prepare a development bath using Microposit™ 351 developer. Dilute the developer to a five to one ratio solution; distilled water to 351 developer.
    - Develop platform batches for two minutes by fully immersing the batch into the solution described above. No agitation is required. Once two

minutes has elapsed, remove and place into a distilled water bath, (circulating water), for two minutes.

- Dry platforms using N_2 gas. Soft bake on hotplate at 110°C for two minutes to ensure all moisture has been driven off.

- Etching. Purpose of the etching step is to remove the areas of silicon dioxide unprotected by photoresist and thereby expose the silicon underneath. The chemical reaction consists of silicon dioxide being attacked by hydrofluoric acid to produce hexafluorosilicic acid: $SiO_2 + 6HF \rightarrow H_2SiF_6 + 2H_2O$. Caution, always use plastic beakers and graduated cylinders for etchants containing HF to avoid damaging lab glassware. Ensure appropriate personal protective equipment is worn and others are aware that HF is in use.
    - Before etching, the platform must first be mounted to a sacrificial slide which will protect the non working surface, not covered by photoresist, from being damaged by the hydrofluoric acid. If this nonworking side were damaged, diffraction of the laser during heating would impact the thermal model. Mounting is accomplished by placing a modest amount of 1818 photoresist on the sacrificial slide and then centering the platform atop the slide; working surface up. Compressive forces will help to spread the 1818 across the entirety of the platform. Care should be taken not to use too much 1818; any excess should be removed from the edges of the mounted platform prior to drying and heating. Soft bake using hot plate at

110°C for two to four minutes to dry and firmly adhere; slides take longer than coverglass.

- This step should be started within 30 minutes of the completion of soft bake. Prepare a fresh Buffered Oxide Etch (BOE) solution consisting of seven parts 40% NH_4F and one part 49% HF in a large Teflon® beaker.
- Place five platforms into the basket sample holder. Immerse in the BOE solution for 15 minutes. Use a stop watch to time the etching process. The etch rate at 22° C is 1200 A/min (Starman, 2008). Note, little variance was discovered between etch rates and effectiveness when comparing slides with coverglass.
- Remove the sample holder from the BOE solution and immerse in a circulating bath of distilled water for five minutes.
- As a final check, view the etched areas of the platforms under the microscope. Repeat etching step in short time increments, no more than five minutes, until sufficient well quality is achieved.
    - Soak platforms in acetone until photoresist dissolves sufficiently to allow the removal of the sacrificial slide. Using the spin coater, clean both sides of the platform using acetone and methanol. Dry using $N_{2.}$

- Cleaning (Post Cleanroom Operations)
    - Begin by mechanically swabbing each platform with ethanol, on both sides, to remove any remaining residue from the clean room processes or large particulates.
    - Sonicate ECS's in ethanol for 10-15 minutes; batches of five or six worked best. Allow to remain submersed in the ethanol until approximately ten minutes prior to use/population. The reason for continued submersion is prevent opportunistic hydrocarbons or water molecules, found in the lab's ambient air conditions, from binding to the wells.
    - Remove from ethanol, allow to air dry under the biosafety cabinet for biological population or the gel dessicator for carbon population. Minimizing the amount of time between ethanol submergence and population is important to mitigate contamination and facilitate a successful population. Never populate while the ECS remains wet with ethanol.

3.2.2 Black Body Production

Black body irradiator is produced in three major steps: Population, Polymerization, and Densification.

- Population
    - Transfer the slides directly from the desiccator to a glove bag under positive N_2 pressure.

- Transfer carbon black directly from satellite container via spatula to the working surface of the slide. Using a machined Teflon© block, (dimensions 1x1x3") work the material in to the wells using downward pressure and multi directional shearing movements; circles, back forth, etc. This action should remove the majority of the excess material from the slide while populating the target wells. Note: Chemical fume hood can cause a particle gradation by blowing/sucking off the finer particulate matter from the carbon black stock.
- Gently remove slides from glove bag without tilting; working face remains horizontal. Using a wipe, clean the backside and edges of the slide. Using the edge of a coverglass, gently swipe the slide to remove surface laid debris. Ensure the edge of the coverglass is cleaned using a methanol swab between uses and replace often to ensure the best edge possible for this step. The methanol cleaning of the coverglass serves to clean debris as well as remove the built up static charge caused by the materials' friction.
- Assess well population distribution using microscopy. If distribution exceeds a mean of 85% or better, proceed to 'Polymerization'. If not repeat 'Population' steps listed above.

- Polymerization. Polymerization occurs in three steps: Sol Gel Production, Application, and Densification/Annealing.
    - Sol-gel Production.
        - Calculate water required. Example below shows the actual dilution with two parts water as determined through trials:

Equation 3. Organosilane Dilution

$$\frac{5\text{g}}{\frac{132.24\text{g}}{\text{mol}}}[Dimethoxymethylvinylsilane] * \frac{18.015\text{g}}{\text{mol}}[H_2O] * 2\text{mol}[H_2O]$$

$$= 1.361\text{g}[H_2O]$$

        - Solution 1: Combine 5g Dimethoxymethylvinylsilane with 15mL of ethanol 95% (EtOH). Cover and stir.
        - Solution 2: Mix 5mL of EtOH with 1.361 g of H_2O and one drop of concentrated Nitric Acid. Stir thoroughly.
        - Add Solution 2 drop by drop to Solution 1 while stirring. Once complete transfer of Solution 2 has been completed, continue to stir for 15 minutes --no heating necessary. Note, cold store when not in use.
    - Application
        - Spin coat each platform using a Laurell Technologies© WS-400B-6NPP-LITE spin processor, or comparable spin coater. Using a glass pipet, apply to the center of the platform approximately 2.5mL of the diluted organosilane per platform.

Deposition of the solution should be finished just as the platform begins spinning. A balance must be struck between flooding the platform so quickly that carbon is washed out of the wells and too slowly where the organosilane begins to cure unevenly. Slides should be spun for 30 seconds at 4,000rpm to achieve an even coating.

- Using microscopy, assess complete coating of platform and wells.

- Densification/Annealing
  - Soft bake at 150°C for 15minutes immediately followed by a ramp up to 450°C over the course of an hour in the box furnace; ramp up in 25C per minute. Do not approach or exceed the glass transition temperature, 573°C, associated with soda lime glass. Allow slides to cool in oven as it cools back to 150°C or less before removal; damage to sample could occur by the simple act of touching the glass with a spatula. Allow to cool at ambient conditions for 30-45 minutes prior to removal and subsequent applications with spin coater.
- Repeat ‘Application’ and Densification/Annealing’ steps once more for a total of two coats per platform.

3.2.3 Biological Sample Preparation

Biological samples are produced in four major steps: Growth/Sporulation, Washing, Serial Dilution, and Population. Most of the aforementioned steps are performed in a Napco© Class II Type A/B3 Biosafety Cabinet.

- Growth/Sporulation. Appendix A outlines the recommended sporulation protocol for use in future work. This work utilized a modified version of that protocol merely due to materials and equipment availability.
    - Prepare agar in accordance with manufacturer's guidance. Agar should cure for a minimum of 24 hours prior to use.
    - Using a sterile loop, transfer one loop full of dried stock sample to culture dish containing cured nutrient agar. Transfer should occur under the Bio-Hood using a sterile spatula. Caution should be taken regarding the placement of the stock and receiving test tube relative to the Bio-Hood ventilation points due to the ease at which the dried spores are moved by air currents; especially the stock *Bt (Toast)* with added flow enhancers. Care should be taken to minimize agar exposure to air and other potential contaminants. Use a zig-zag pattern of application to help in determining contaminant growth once sample has begun germination.
    - Following inoculation, place culture dish in the incubator, (Labline® Imperial III Incubator), for 48hrs at 34°C to germinate and begin sporulation. Can grow for seven days, but would not recommend any longer due to drying of the agar.

- Prior to harvesting, confirm sporulation has occurred using a magnification equal to or greater than 100x under phase contrast lighting. Spores should be phase bright. If spores are not apparent, incubate culture longer – checking for phase bright spores every two hours. See Appendix A for a Brook's Sporulation Protocol.

- Washing. Intent of the washing step is to refine the sample. Following growth and harvesting, the sample will contain spores, vegetative cells, agar and associated bio debris. In order to avoid populating the etched platforms with this bio debris, and not the intended spores, a washing must take place.
  - Pipette sterile water onto the culture plate. Sufficient quantity should be used to completely cover plate surface.
  - Bathe a bent steel spreader in methanol and immediately follow removal with flame sterilization. Gently dislodge the biological growth, germinated *Ba* or *Bt*, from the agar using the sterile steel spreader. Place the plate atop a piece of equipment, i.e.: cell scraper, to help pool the slurry to one side.
  - Using a sterile pipet, transfer the entirety of the slurry in to a 15mL test tube for centrifuging. Using a sterile pipet, transfer approximately 1,500µL of sterile Millipore water to the sample. Total volume of the sample should be between 5-10mL.
  - Since the centrifuge step occurs outside of the biosafety hood, ensure the exterior of the tubes are wiped with methanol to avoid contaminating

laboratory equipment. Label after sterilization ensuring organism type, sporulation temperature and growth time are included.

- Vortex for one minute using a Daigger© Vortex Genie 2 at the highest setting; eight.
- Centrifuge for 20 minutes at 4,000 revolutions per minute (rpm) and 4°C in an Eppendorf© 5810R Centrifuge. Ensure balance by filling ballast test tubes to same level as their corresponding sample tubes.
- Pour off supernatant taking caution not to disturb the spore pellet.
- Repeat the last three steps two more times; for a total of three wash/centrifuge cycles. Following the last centrifuge cycle, pour off supernatant and refrigerate pellet at 4°C.
- Silicone Removal. If the sample being washed has any additives that are insoluble in water, (in example the silicone flow enhancers of *Bt (Toast*)), then the second wash should be conducted using a 70% ethanol solution. Take efforts to minimize contact time.

- Serial Dilution. A serial dilution helps to quantify the sample by calculating the starting population density of the, now concentrated, spore sample. Because the starting concentration is likely so high that any attempts at growth counts would result in continuous growth across the culture plate, several dilutions of the starting stock are made and assessed. Based on the counts of these dilutions, an inference is made as to the population density of the original sample.

- Label ten test tubes. Recommended naming convention is $SD10^{-1}$, $SD10^{-2}$, $SD10^{-3}$…..$SD10^{-10}$.
- The sample to be quantified should be found in pellet form following the 'Washing' step. Using sterile pipettes, add sterile water to the sample bringing the total volume to 10mL. Label as 'Stock' sample. Vortex for one minute.

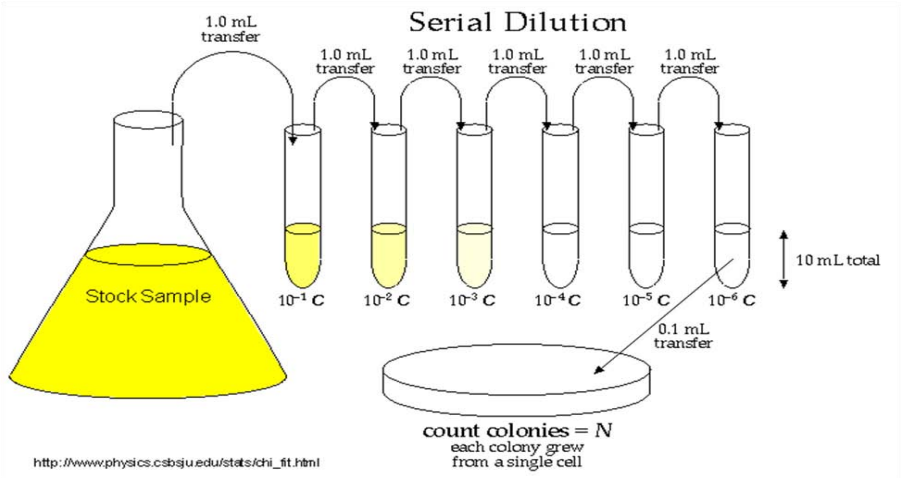


Figure 11. Serial Dilution Schematic

- Transfer 1mL of original stock solution to the tube labeled SD10. Using sterile water, fill to 10mL and vortex. Repeat this 1mL transfer, filling to 10mL and vortexing step for each successive dilution until the sample $SD10^{-9}$ has been completed; see Figure 11. Quality checkpoints include the use of sterile water to fill tubes up to exactly the 10ml mark, vortexing in between transfers to ensure spore distribution throughout each dilution is uniform, precision pipetting and finally labeling.

- Transfer 1ml of each dilution to a culture plate with counterpart labeling. Use the spin plate to ensure a uniform spreading of sample across the plate. Incubate for 24 at 34°C.
- Assume each bacterial colony is generated by one spore. Observe culture plates and determine which are feasible for counting; any dish containing 30-300 colonies. Samples with innumerable CFUs were labeled as having continuous growth, all other were recorded with the number of CFUs counted.
- Apply the following equation to dilutions meeting the protocol criteria to determine population density, (see Equation 4):

Equation 4. Population Density

$$?\left[\frac{CFU}{mL}\right] = \frac{\#\ CFU}{(df * Volume\ Plated\ [mL])}$$

CFU=Colony Forming Units
df = dilution factor; ie: 10^{-5}
Volume Plated = in mL

In example, if the culture plate inoculated with 1mL of sample from the dilution SD10^{-7} yielded a CFU count of 286 colonies, the original spore solution would be said to have approximately 10^{9} CFU/mL. This calculation was executed for each plate observed to have between 30 and 300 colonies. An average was used of the results to determine an estimated population density for the stock sample. It is assumed that the calculation is accurate to the order of magnitude.

- Population. Previous population efforts relied on the use large quantities of spores principled on high spore to surface area ratio and evaporative forces. Challenges with this method included a large number of excess spores deposited on the glass surface outside of the sample wells. This surface deposit causes challenges during post treatment growth and counts. The technique outlined below relies heavily on evaporative forces, but also draws on gravitational and shearing forces to ensure a thorough population. The challenge of surface deposits is mitigated by using a, comparably, low density sample.
    - A target population density of 10^4 was estimated for use during population. This value was based on the target number of spores per well; roughly 5-10 spores per well at 532 wells. For the *Ba* sample used in this study, the stock population density was 10^9 CFU/mL, therefore, dilution used for the initial population runs was $SD10^{-5}$. Final dilution used data runs was $SD10^{-2}$. Recommend population trials to determine best dilution. Based on experimental criteria, a tradeoff must be made between total number of wells populated versus number of spores per well. Higher concentrations result in a greater percentage of wells populated, but many wells exceed the target population or have excessive surface deposits.
    - Remove an etched coverglass platform (ECS) from ethanol and place it atop a flat, absorbent material and allow air dry. Working side should be placed upwards. Kimwipes® or other low or lint-free material is used to absorb excess sample material preventing it from coming in to contact

with the back side of the slide; causes microscopy and contamination challenges later.

- Vortex the sample and then transfer 250µl of sample to the working surface of the ECS. Spread the sample evenly across the entire ECS surface using the tip of the sterile pipet used to transfer and/or a rocking motion. Though sample should travel all the way to the edges of the ECS, care should be taken not to contaminate the back side of the ECS; nonworking surface.
- Allow to air dry for five minutes or until sample begins to pool and separate from one uniform coat. Use a sterilized piece of coverglass to perform multiple squeegees, wipes, of the ECS surface. This action utilizes compressive and shearing forces to further deposit spores inside of wells while removing surface deposits. Wipes should be done in both directions and the coverglass edge should be rotated and cleaned between each effort. Caution should be taken not to execute this step too early in the drying process for fear of causing an uneven deposition and not affording sufficient time for evaporative and gravitational forces to take effect. Also, the coverglass will score the ECS if too much force is used.
- Once completely air dried, confirm population and cleaning using microscopy. Once dry, deposited spores are difficult to remove from the glass surface of the ECS. A modest amount of sterile water can facilitate further cleaning. Subsequent hydrations or reapplications of sample do

not pose the same level of depopulation risk as seen in the carbon populations.

- Storage. Store all populated slides in Dessivac® containers at 4°C. Dessivac® containers use a sealed gel desiccant vice the traditional granular desiccant; mitigates contamination concerns.

3.2.4 Sample Configuration

Original intent was to use a 5 µm well for bio and 50 µm well for carbon. Population and microscopy challenges using the 5 µm resulted in the use of 50 µm mask design for both etched platform requirements; bio and carbon. The 5µm well proved less difficult to populate, but extremely difficult to qualify and quantify population. Multiple attempts were made at aligning the two types of platforms to ensure and exact alignment of the biological well atop that of the slightly larger carbon wells. Even with the use of the same photomask, the end product variance proved too great for the planned configuration. At any one time only half of the trial sample could be properly aligned. Theoretically the alignment failure is due to variance in well size and spacing. This variance could be caused by either variance in the mask design itself, or in the exposure of the photoresist during platform production. A 0.25µm tolerance is listed in the photomask certification by the manufacturer; Photosciences©. This relatively small error, propagated across all 38 columns of each platform causes a tremendously challenging alignment problem. Even with the favorable size ratio between the biological and carbon platforms, roughly 1:2 biological to carbon, alignment would fail after moving just six to seven wells beyond an aligned quadrant of wells. It is possible

that the EVG620 substrate thickness settings caused a variance in the final platforms. For the coverglass exposure, the substrate thickness is 0.2mm while during slide exposures it is set for 1.1mm. This substrate thickness dictates the contact distance allowing a soft contact between substrate and mask during exposure. Finally, the simple act of flipping one of the platforms, to ensure working surfaces are facing one another, could be enough to throw off evenly identically made platforms. If the error is in the distance between wells, in any two directions, then alignment would prove impossible—platforms are not a mirror image. The below listed is the final protocol used during alignment and final sample configuration.

- Alignment
    - Ensuring that both working surfaces are facing one another, place the two platforms in contact. Using the L-brackets, visible by the naked eye, roughly align the two platforms.
    - Using 100x magnification, align the L-brackets under microscopic observation. This step serves to calibrate the researcher as to the range of focus to traverse between mediums.
    - Scan to the center of the ECS and obtain an objective view which includes four wells simultaneously. Using gloved fingertips, adjust the ECS until all four wells are completely covered by counterpart carbon wells. This step proves much more difficult than it reads. Techniques using tweezers, putty, and tape for stabilization and movement control were attempted to

overcome overcompensation. Technique practice and refinement dictate the ease of micro adjustments.

- Adjust through the depth of the four wells to determine the extent of centering between the two mediums. If satisfied, traverse across the sample and repeat observation in depth using four different wells. Commonly it was possible to align four wells, only to move just five well in any one direction and observe misalignment. Best practice, using this platform design, is to achieve alignment of approximately half the sample. Record which half of the sample is aligned and will be treated.

- Stabilization
  - Using a small piece of Scotch® tape and a pair of tweezers place the tape across the two mediums; the smaller biological ECS will be atop the black body slide. Technique should be used so that the tape makes contact with the slide first and allows gravity to place it in contact with the ECS. Once in contact, use a sterile swab to fully flatten or fix the tape to the two mediums.
  - Reconfirm alignment. If alignment still holds continue by placing a 'gator clip', metal clamp, to the sample. Placement of this clip should occur at the end away from the aligned wells.
  - Reconfirm alignment. Apply 2-4 more pieces of tape to the end of the sample; opposite that of the clip.
  - Store all final sample configurations in Dessivac® containers at 4°C.

Characterization. Sample characterization is the qualification and quantification of all the wells within the sample area. This is a multiphase process and occurs prior to thermal treating, before growth and after growth. Carbon population counts occur prior to heating since many wells are partially destroyed during thermal treatment. Spore counts occur only after separation of the two platforms. Early trials showed that there is some transfer of spores between the two mediums while in contact. Counting spores after separation mitigates false positives; spores characterized as killed when they were merely removed or moved. Each carbon well was evaluated for suitability by estimating percentage populated; any well with 85% or more populated was deemed acceptable. Only carbon wells found within the sample area were assessed. Naming convention is outlined in paragraph '3.2.5.2 Counts', but carbon well naming was driven by the biological wells in the sample area. Carbon wells that corresponded to these biological wells were assigned well names respectively. This means that, theoretically, a carbon well in the middle of the black body platform could be called well A1 due to its alignment with the biological well A1—nearest to the edge of the biological platform.

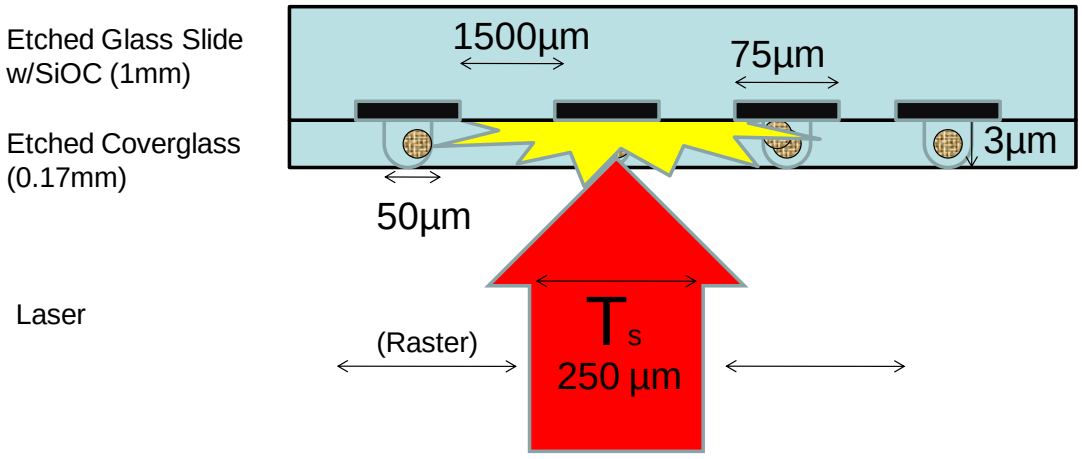


Figure 12. Trial Sample Configuration and Dimensions. Not drawn to Scale

3.2.5 Thermal Inactivation

Spores are heated against black carbon wells using a solid state laser (Nd: YAG). The target temperature range is 1000-1600°K for times of milliseconds to three seconds. Heating temperatures were varied by using different laser powers. The heating times are controlled by adjusting the raster rate of the sample relative to the laser beam. The thermal radiation from the carbon well was measured using a Fourier-Transform Infrared (FTIR) spectrometer with a sensitive Mercury Cadmium Telluride (MCT) detector, from which the temperature was obtained by fitting a Planck function to the infrared spectrum from carbon.

- Sample Alignment
    - First step before mounting to sample holder is to confirm alignment of the biological sample with that of the carbon black body slide again. Even the slightest mishandling during storage or transfer to the laser can cause misalignment.
- Mounting Sample Holder
    - The sample holder has a 13mm circular aperture through which samples are exposed to the laser. Center the sample atop this aperture ensuring the target area is within the aperture. This opening roughly affords exposure to 48 wells; 6 rows by 8 columns.
    - An alignment mark is etched in to the sample holder. This etch mark indicates the laser's zero in respect the sample holder. Using a

microscope, ensure at least three successive sample wells are located directly over this alignment etch, (see Figure 13).

- Using tape or compression clips, secure the sample to the sample holder. Once confident that sample is fixed, reconfirm well alignment with sample holder alignment etch.
- Secure sample holder with sample in to spectrometer apparatus. Note, biological ECS should face the laser origin—as if the laser will irradiate through the biological well to heat the carbon.

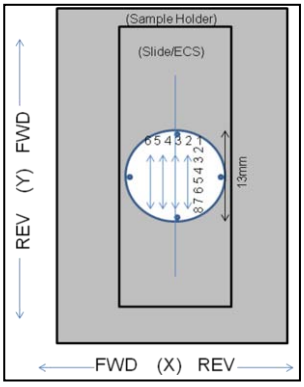


Figure 13. Sample affixed to sample holder. Diagram of rastering pattern with control settings and dimensions.

- Laser alignment
  - Put on safety glasses. Set laser power to 0.2W. This step requires the raster controls, paper covered paddle and a handheld ultraviolet (UV) lamp. Place the paddle in the apparatus as a backdrop to the sample. Illuminate with the paddle with the UV lamp. Caution, do not allow the UV lamp to irradiate the sample. Though the power of the handheld lamp

is relatively low, this premature exposure by an alternative inactivation strategy could impact the results.

- Using the max raster rate, scan the sample anywhere in the aperture, both X and Y directions, until a circular shadow is cast on the paddle. Slow the raster to approximately ¼ turn. Using single clicks of the raster control, refine the shadow until its edges are a clear as possible. Lowering the lights in the room may assist in this step since observation of the diffracted light is difficult given the low laser setting, (see Figure 13).
- Once centered atop a well, increase the raster rate and scan in the X direction. Researcher should be able to observe similar circular shadows in equal increments along the sample column. This confirms the laser alignment with the sample.
- While maintaining alignment atop that column, scan in the X direction until the far light limit is achieved. An audible signature from the sample stage motor can be heard as well as visual scale showing the stage position relative to total travel distance. Lower the raster setting and reverse directions until centered atop the first well encountered from the edge.
- Change the raster controls to the Y setting and scan upwards (FWD) to the top of the sample; raster terminus. Complete the last few millimeters of this movement at the setting to be used during exposure. At the slowest raster rate, the difference between moving at the target raster speed and not moving at all is a very fine adjustment on the raster control dial.

    - Apparatus is now configured to expose the first row.

- Exposure
    - Set the laser power to desired setting; all trials for this research were conducted using 1.6W.
    - Using a mechanical clamp depress the raster control to move downwards (REV). Clamp is helpful to allow researcher to move into viewing position to confirm raster rate or to execute a well count during exposure.
    - Once the first scan is complete, repeat in the opposite direction by merely reversing the clamp (FWD). Three scans total should be executed per row.
    - Once three scans have been executed for the row, reduce the laser power to 0.2W. Increase the raster rate and center the laser atop a well, any well, along the row just treated.
    - Switch raster control to X and begin depressing the FWD control button in a consistent, repeatable manner. Keep track of the number of depressions, 'clicks', it requires to achieve the next row. This value should remain consistent for the remainder of row to row transitions; given a uniform sample configuration.
    - Once the next row is achieved, refine the center using slow raster, fine adjustments in both X and Y directions if necessary. Once centered, raster upwards to the top/beginning of that row. Complete the last few millimeters of this movement at the setting to be used during exposure.

- Repeat all ‘Exposure’ steps listed above until all six rows have been exposed. Caution should be taken throughout the process to ensure proper laser power and raster rate is set during alignments and during exposure. Do not align at a power greater than 0.2W.

3.2.6 Determination of Viability

3.2.6.1 Incubation

Following thermal treatment and post treatment counts, place the samples into Criterion® ‘Nutrient Agar: for cultivation of nonfastidious microorganisms’. The working surface of the ECS should be down; in contact with the agar. Care should be taken to ensure the sample is close enough to the edge of the culture dish to ensure a full range of raster by the microscope sample stage. Main ingredients for agar used in this study include: agar, gelatin peptone, and beef extract. Agar should be cured for 1-2 days prior to use to ensure moisture content is acceptable. Agar that is too moist can cause the mobilization of spores and vegetative cells; both in to and out of wells. Too dry and growth rate may be retarded due to desiccated conditions. Dry agar also increases the chance that air/gases may be trapped between the sample and the agar. These gas pockets prevent the spores from exposure to the nutrient required for growth. Samples should be incubated for 3 hours in an incubator set at a temperature of 34°C. Once this initial incubation period has elapsed, growth will continue at ambient laboratory conditions (approximately 20°C). A more dilute sample during population will reduce this challenge, however, the total number of wells populated will be reduced. A balance must

be met between number of spores per well, total number of wells populated with an acceptable number of spores (1-15) and the amount of excess spore material outside of the wells. This spore debris deposited on the ECS surface outside the wells can prove detrimental during post treatment and viability counts.

3.2.6.2 Counts

Post treatment counts for this study were executed between three and nine hours from time of incubation start. Normal growth can be observed at just over an hour. Hawkins showed that germination can be retarded following thermal treatments as the spore's DNA undergoes repair once germination begins. In her trials Hawkins showed that spores treated with as low as 177ºC required three times the duration to begin germination compared with that of untreated spores (Hawkins L. S., 2008). Each well was observed prior to incubation and after three hours of incubation; or as shortly thereafter as possible. Photo documentation of the individual wells was executed with assignment of an alphanumeric naming convention to distinguish between them.

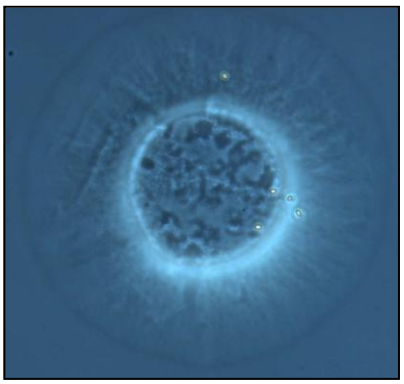


Figure 14. *Ba* Populated Well. Counted as a three spore population density though five are shown.

Using perimeter markings applied during the thermal treatment to outline which area of the ECS was thermally treated, wells were thoroughly characterized. Alignment of the carbon well with that of counterpart biological sample wells was confirmed. Each carbon well was evaluated for suitability by estimating percentage populated; any well with 85% or more populated was deemed acceptable. Each biological well was observed and spore counts were completed. Wells containing 1-15 spores were deemed acceptable, (see Figure 14). Wells with more than 15 spores were difficult to observe throughout the depth of the well; counts were not repeatable with the level of accuracy needed.

Determination of viability is done using a microscope capable of 400x magnification under phase contrast lighting; for this study a Zeiss® Axioskop Routine Microscope was used. If a spore remains viable and conditions are met for it to germinate, the outgrowth will initiate from the spore's original location. As the cell replicates and divides it does so from the terminus. This unidirectional growth makes the determination of a single spore's germination, or failure to germinate, possible. Each chain of *Bacillus* links is a distinct and separate genetic line originating from one spore. In early stages of germination chains grow in a generally straight line. As crowding occurs chains will turn and sometimes fold back atop themselves making counting more difficult; if not impossible. Each organism used in this study, *Ba(Sterne)* and *Bt(Kurstaki)*, displays a different growth rate. These growth rates are less a function of the organism and more that of the sample's original preparation and the environmental conditions of that preparation; temperature, moisture content, pH. Ideal growth times,

based on the organisms used, were between 3-5 hours. This statement is based on a sample dilution used during the work; population density of 10^7 CFU/mL. If multiple counts are planned, one hour intervals up to six hours work best. Counts per row can take upwards of one hour, but as short as 20 minutes if photo documentation of each well is the goal; with counts occurring later. After six hours, vegetative cell growth can become overwhelming making it difficult to execute the counts. Cells originating outside of wells begin to encroach into the sample wells complicating efforts to determine their point of origination.

3.2.7 Sampling Design

Intent of the original platform design was to maximize the number of samples per individual etched coverglass; 532 wells. This affords the researcher the ability to select a number of treatment and sampling plan options per platform. The original intent was to use a systematic sampling plan to help mitigate microscopy challenges. Using a 5μm well, identifying an individual well with a high degree of repeatability proved very difficult making simple random sampling impractical. This proved especially true after any population attempt, carbon or biological, due to debris outside of the wells. Identifying rows, however, was possible. In systematic sampling, samples are evenly spaced after a random start position is chosen (Christian, 2003). Due to the population challenges encountered during black body and biological sample production, the number of wells per platform available for treatment and observation was decreased. Success for black body production was that 85% or more of the wells were populated with 85% or more carbon per well. Success for biological sample population was 1-15 spores per well

with greater than 85% of wells populated. Because of low population percentages for both, the original sample size of 532 wells was cut approximately in half. The decision to move towards a 100% sampling of the acceptable wells was made based on this fact and the target confidence interval. Calculations indicated that to achieve a 90% Confidence Interval, with an error of estimation of 0.05, approximately 271 samples were needed, see Appendix C.

When the final sample configurations were achieved, it was determined that due to the cumulative error in well alignment perfect alignment of the two platforms could not be achieved. Efforts proved that only half of the sample was able to be aligned, therefore, treated at any one time. This further reduced the number of wells per trial to a range of 48-71. The decision to define individual spores as a sample was made to maintain a statistically significant sample size. Samples were now defined as individual spores with success being defined as a nonviable spore; a spore that failed to grow following three hours of incubation and variable time at ambient conditions during counts.

Systematic sampling was used in early population trials to determine distributions for the population techniques used in the work; both carbon and biological. Due to the time required for each count/assessment and the total number of wells a systematic sampling plan was used. Every other row was counted in its entirety. This sampling plan was selected to provide information regarding total population of the platform as well as row/column trends across the sample.

4. Analysis and Results

4.1 Results of Carbon Population Trials

Population of the 50µm etched wells with carbon black proved to be very challenging. Variance in well quality, cleanliness of the platform, particle separation of the carbon black, and care in handling all contributed to population challenges. Appendix D outlines the numerous lessons learned over the course of three months of trials focused on producing black body platforms with a greater than 85% population of wells with an acceptable amount of carbon. Success was defined as 85% of the wells per platform had to be populated with carbon deposited in at least 85% of wells' surface area. Only acceptable wells were used during thermal treatments. Table 4 shows the results of four distribution counts executed to validate the black body production protocol. The column 'Mean-Total Carbon Population' provides an average population of total well surface area per platform. This means that between 61 and 78% of the 532 wells, per platform, were populated with carbon. Of those 532 wells, between 57 and 71% of those

Table 4. Carbon Population Data

	Sample Size(N)	# Viable Wells	% Viable Wells/Platform	Mean-Total Carbon Population	Standard Deviation	Standard Error of Mean
Slide 25	227	129	56.83%	61.23%	0.4545	0.0302
Slide 24	228	158	69.30%	74.58%	0.3677	0.0244
Slide 31	228	167	73.25%	78.75%	0.3573	0.0237
Slide 29	228	161	70.61%	74.34%	0.4038	0.0267

were deemed acceptable for use in the heating trials; see '%Serviceable Wells/Platform'. Figures 15 and 16 show the mean, serviceable carbon population by row and by platform respectively. The intent of the by row comparison is to show any increases or decreases

in progression across the platform. The population technique followed by a swiping cleaning technique, targeting any deposition outside of the wells, would lead to concerns regarding an irregular population favoring one side of the platform. As shown, there is little evidence that speaks to a lopsided deposition on one side of the platform or the other. The relatively low percentage of acceptable wells per platform indicates that success or failure of the population per well is dictated by the quality of the well itself.

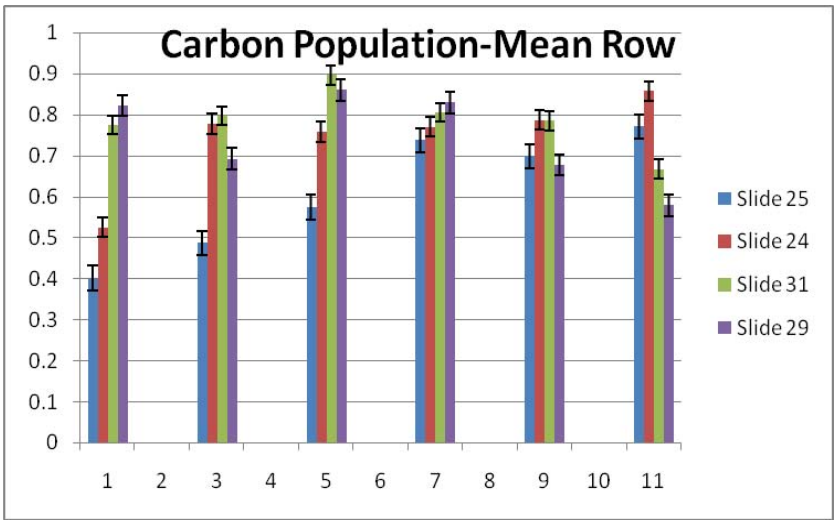


Figure 15. Average Carbon Distribution by Row

It was not uncommon for a completely populated well to directly neighbor a well with no carbon present at all, or less than 25%. This fact speaks to irregularity in the individual well's receptiveness to the carbon. Possibilities include large variance in the quality of the glass which in turn created variance during the etching process. Another theory is that the application tools used, were not uniform enough at the microscopic level to ensure a uniform application; Teflon block and glass coverslip for shearing and compression forces. Efforts to mitigate this concern were taken which were included in

the methodology above. Finally, obligation of the wells' available bonds by water molecules or opportunistic hydrocarbons could have impacted population success. This obligation is possible though unlikely due to the ethanol wash, storage of unpopulated platforms and depositions under an N_2 environment.

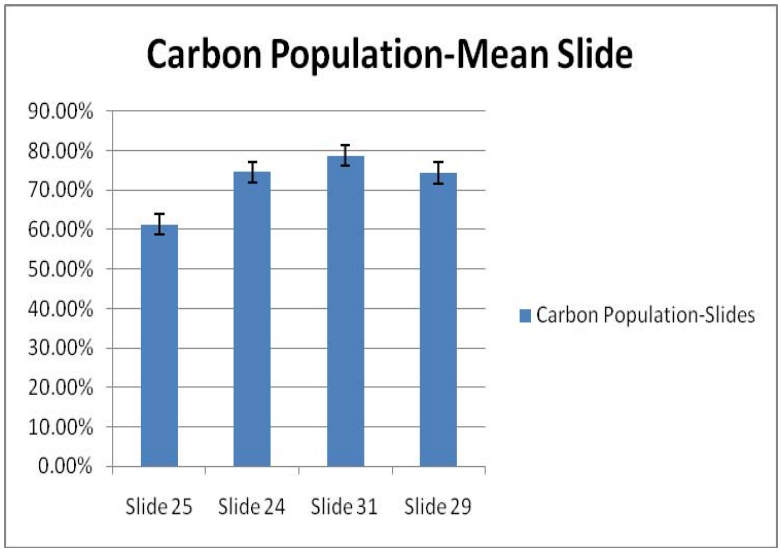


Figure 16. Average Carbon Distribution by Platform. Total well surface area populated with carbon for entire platform.

Observations of note. A large variance was observed during trial runs to refine the protocol. A larger than expected variance was observed both on the same slide as well as within platform batches produced and then populated using the identical techniques. Wells were commonly observed that were completely populated while others, just 1.5mm away, remained completely unpopulated.

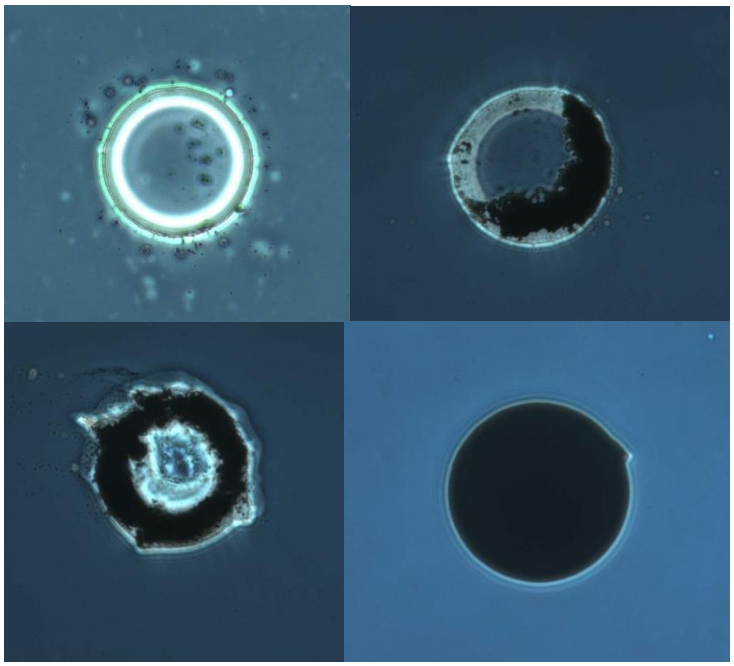


Figure 17. Carbon black population. Wells showing various states of population. Bottom right is final product; post polymerization.

Once any form of organosilane coating was added to the black body platforms, (whether it be via vapor deposition, as part of a carbon black slurry, or a spin coating), those wells then became unreceptive to future population attempts. If the first population was insufficient, all further deposition attempts ran just as much risk of depopulating the well as it did of populating it. In example, successive swipes using the coverglass to clean excess material from the glass surface would depopulate wells by creating a static charge—macro particles could be physically observed leaving the black body platform and attaching to the cleaning tool. Final well population protocol resulted in a micro-deposit of carbon black larger than 50µm. The roughened glass area immediately surrounding the well was receptive to the carbon black; arguably more so than the defined 50µm well. Final black body size ranged from 50 to approximately 75µm. Final

black body well configuration, full deposit with polymerization, was insufficient to withstand target temperatures. Wells were partially destroyed at laser settings of 1.6W, however, no evidence of destruction was observed at 0.8W. Shrink cracking of the black body irradiator was observed and the transfer of carbon particles to the corresponding biological wells occurred.

4.2 Results of Biological Population Trials

Population of the 50µm etched wells with spores proved comparatively easy when viewed against the population of a 5µm well. Variance in well quality, cleanliness of the platform, and spore population density all contributed to the population trial's success. Appendix E outlines the numerous lessons learned over the course of study regarding biological sample production. Success was defined as 85% of the wells per platform populated with at least one spore, but no more than 15. It was subjectively determined that 15 was the maximum number of spores per well that could be reproducibly observed and assessed for viability. Table 5 shows the results of four distribution counts executed to validate the biological population protocol. The column, 'Mean-Total Spore Population' shows the average number of spores per well by platform. This data is graphically displayed in Figure 18.

Table 5. Ba Population Data

	Sample Size(N)	# Viable Wells	% Viable Wells/ Platform	Mean-Total Spore Population	Standard Deviation	Standard Error of Mean
ECS04	204	165	80.88%	5.16	7.5259	0.5269
ECS15	204	175	85.78%	3.75	5.7752	0.4043
ECS19	202	127	62.87%	9.69	15.0384	1.0581
ECS20	204	176	86.27%	5.91	6.3224	0.4427

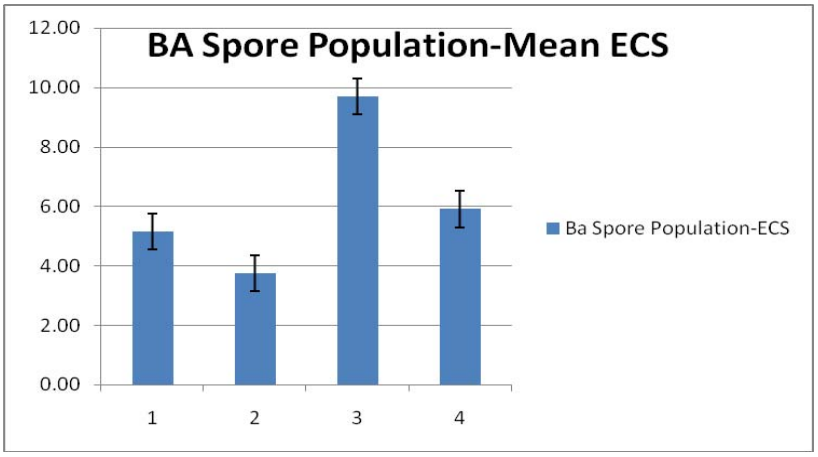


Figure 18. Average Ba Spore Popualtion per platform. Ratio of total number of spores deposited and number of wells sampled.

The conclusion drawn from these figures is that even though the larger diameter well was used, the spore deposition per well remained relatively low; between three to nine spores on average. Assuming a uniform slope, the approximate volume of the well is 32,708 μm^3; see Equation 3.

Equation 5. Well Volume. One half volume of a sphere.

$$\text{Volume of the well} = \frac{1}{2} * \left(\frac{4}{3}\pi r^3\right)$$

When the size of the spores, approximately 1um, is compared to the well volume the comparatively low population is indicative of the difficulty in populating the wells. The hydrostatic pressures and challenges of populating a microscopic well with a hydrophobic spore remain a challenge. A balance must be met between the total number of wells populated with a sufficient number of spores versus the amount of deposition occurring outside of the wells. This balance is struck in the form of the starting population density of the sample being applied to the etched platform; for these trials it was a sample density of 10^7CFU/mL. Figure 19, on the following page, shows that the

mean spore population per row increases as the platform is traversed from one side to the other. This increase in spores is caused by the cleaning method used to remove spore deposition outside of the sample wells. Through shearing forces, the coverglass swiping technique further deposits spores in wells as it progresses across the platform. An alternative cause for this is the fact that as spore deposition is occurring, by gravitational and evaporative forces, spores have a tendency to seek the leading edge of the water. Any compressive forces, such as those seen in early population trials using a coverslip to spread the spore sample across the platform, forces the spores towards the edges of the platform.

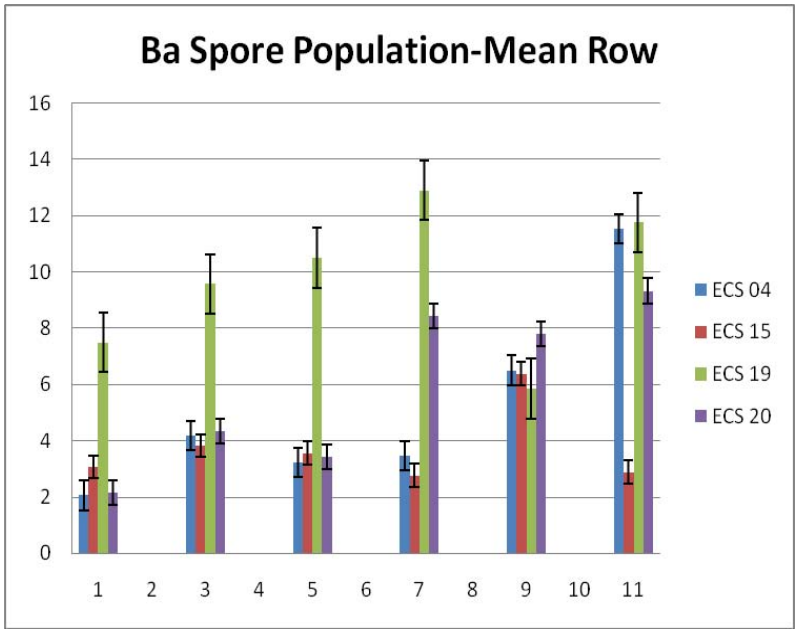


Figure 19. Average Ba Spores per Aliquot. Aliquots are full counts of sample rows.

Since no compressive forces were used in the final protocol, the only other cause surmised could be an uneven platform which resulted in a lopsided deposition due to

gravitational forces. The most likely cause was mechanical, deposition as a result of shearing forces across the slide where spore pooling occurred during cleaning.

Observations of note. Spores would tend to deposit early or they would remain suspended in the water where they remained mobile until the evaporative edge moved to their position. Observations made during population runs showed spores being drawn into nearby wells as evaporation across the platform occurred. The forces most responsible for the successful population of the wells were cohesive and adhesive forces due to water surface tension. Evaporative shrinkage was organized by these forces. Secondary force at work was the gravitational force which slowly deposited spores. Van der Waals forces binding spores to each other and to surfaces also played a role. Spores tended to clump in pairs. Finally, once deposited and dried the spores tended to stay in place when exposed to properly cured agar. If the agar was too wet, however, some mobility of the spores was recorded. Vegetative cells proved more susceptible to increased agar moisture than spores.

4.3 Results of Thermal Inactivation Strategies

The sample configuration used in this study is capable of treating the spores at a temperature at least 72% of the source temperature based on estimates of the average air gap. This estimate was based on the steady state thermal model presented in paragraph '2.5 Thermal Diffusion' which includes only conductive heating, neglecting small contributions of convective and radiant heating. Regardless of cleaning efforts following population of the etched platforms, a significant amount of debris was present on the glass surface outside of the sample wells. The material having the greatest impact on the

air gap between the two samples is the carbon black deposited outside of the wells on the glass surface. Assuming a particulate size of 10µm, the temperature seen by the spore is at least 72.4% of the measured temperature; see Equation 6

Equation 6. % Source Temperature Spore Exposure

Final Sample Configuration:
t_a=10[μm]; t_g=1.15[mm]

$$\mu = \frac{K_g t_a}{K_a t_g} = \left(\frac{1.05}{0.024}\right) \bullet \left(\frac{0.00001}{0.00115}\right) = 0.38043$$

$$T_a(t_g) = \frac{1}{1+\mu} T_s + \frac{\mu}{1+\mu} T_o$$

$$T_a(t_g) = \frac{1}{1.38043} T_s + \frac{0.38043}{1.38043} T_o = 0.275 T_o + 0.724 T_s$$

If $T_s \gg T_o$ then the temperature at the spore is $\approx$ 72% of the source temperature.

Based on Planckian fits to spectra collected during thermal treatments, the temperature emitted at the surface of the black body irradiator was 800K± 120 for treatments at 1.6W laser power. An error between 10-30% has been assigned based on the assumption that all errors are due to the imperfect blackbody absorption; ± 120 shows 15% error. Based on the calculation above, the temperature at which the spores were treated was at least 576K. One data point essential to the model is the size of the air gap; which remains uncertain. Factors responsible for an increase in air gap include carbon particle residue on the glass surface and malformations in the glass itself. Carbon particles were generally observed to at a maximum of 20µm under microscopic observation. These estimations and an assumption of a 10µm air gap result in a spore

heating of approximately 72% of the source temperature. Sample mounting designs that eliminate the air gap are necessary to reduce this uncertainty.

Table 6. Probability of Kill Data

Raster Rate [mm/sec]	Distance [μm]	# Exposures	Total Time [sec]	Power [W]	Temp [°C/K]	Kill Probability	Standard Deviation	Standard Error of Mean
0.41	50	3	0.366	1.6	303/576	71.43%	2	0.2389
0.41	50	6	0.732	1.6	303/576	71.61%	3	0.4209
0.06	50	3	2.500	1.6	303/576	72.32%	2	0.1996
0.06	50	6	5.000	1.6	303/576	77.45%	3	0.3845

Thermal runs were executed using a varying raster rates and a fixed laser power; 1.6W. An increase in probability of kill is observed as function of raster rate and total number of exposures. As the total time of treatment increases so does the level of success achieved by the inactivation strategy. The relationship between kill probability and duration of treatment is shown in Table 6 and Figure 20. The data is difficult to compare to other work found in literature for a number of reasons.

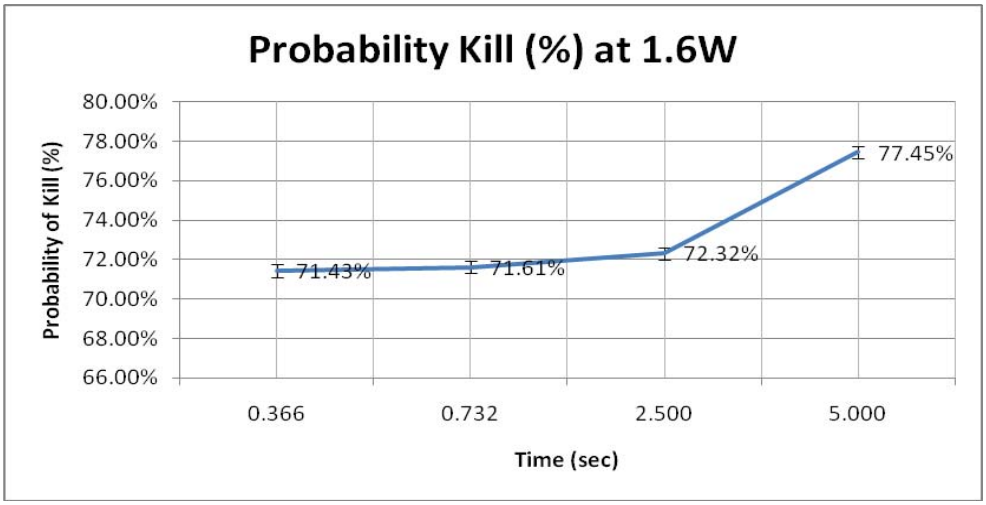


Figure 20. Probability of Kill at constant temperature

First, data at comparable durations is limited, though there is one comparable study from which comparisons can be made; Battelle's© 'Thermal Deactivation of Aerosolized Bacteria' (Alexander, Ogden, LeVere, Dye, & Kohler, 1999). The starting and post treatment population densities used in that study are much larger than the data sets in this study. The measure of effectiveness used in the Battelle© work is presented in terms of Log reduction, (see Figure 21). Starting population densities are on the order of 10^5 thru 10^9. The subsequent reduction, down from 10^9 to 10^7, would then be characterized as a 2 Log reduction.

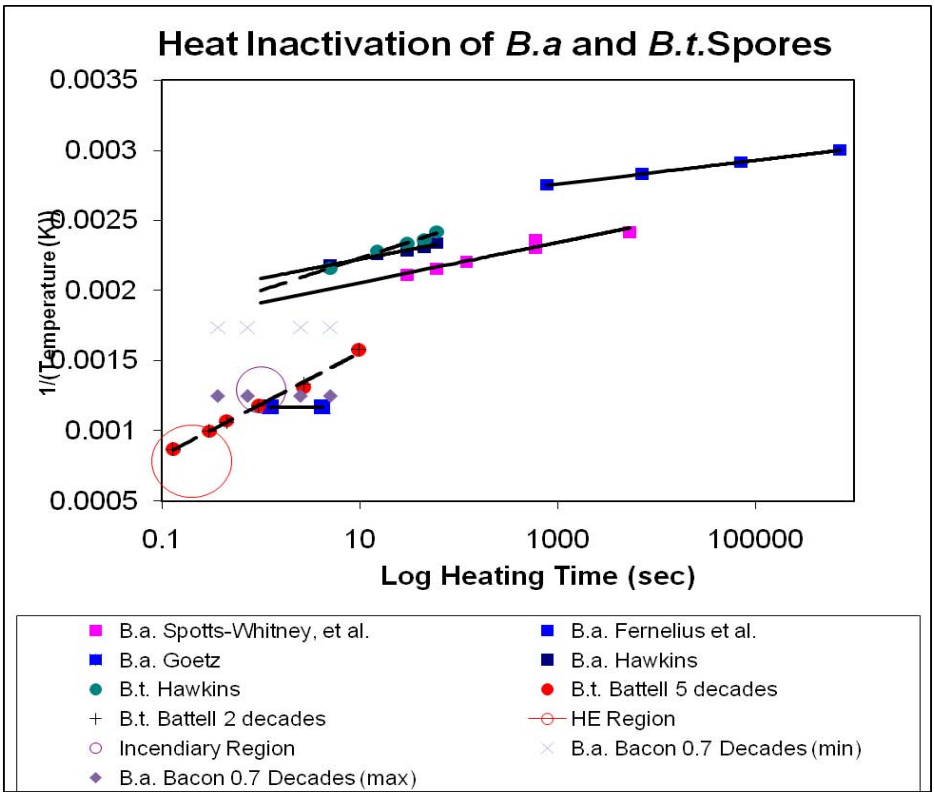


Figure 21. Literature summary of thermal treatment of *Bacillus* spores (Burggraf et al, unpublished)

This protocol does start with a population density of 10^9, however, the population technique is specifically designed to reduce that density to a manageable number of spores resulting in a population density per platform in the hundreds. A simple ratio comparison could be made which is shown in Figure 22. The data point chosen for comparison with the Battelle data was the shortest duration, 0.3659sec, with the lowest probability of kill, 71.43%. The plot was built by interpolating temperature values from Figures 8-13 of the Battelle report 'Thermal Inactivation of Aerosolized Bacteria', (Alexander, Ogden, LeVere, Dye, & Kohler, 1999). These figures showed viability versus temperature data for various exposure durations and temperatures for *Bt*.

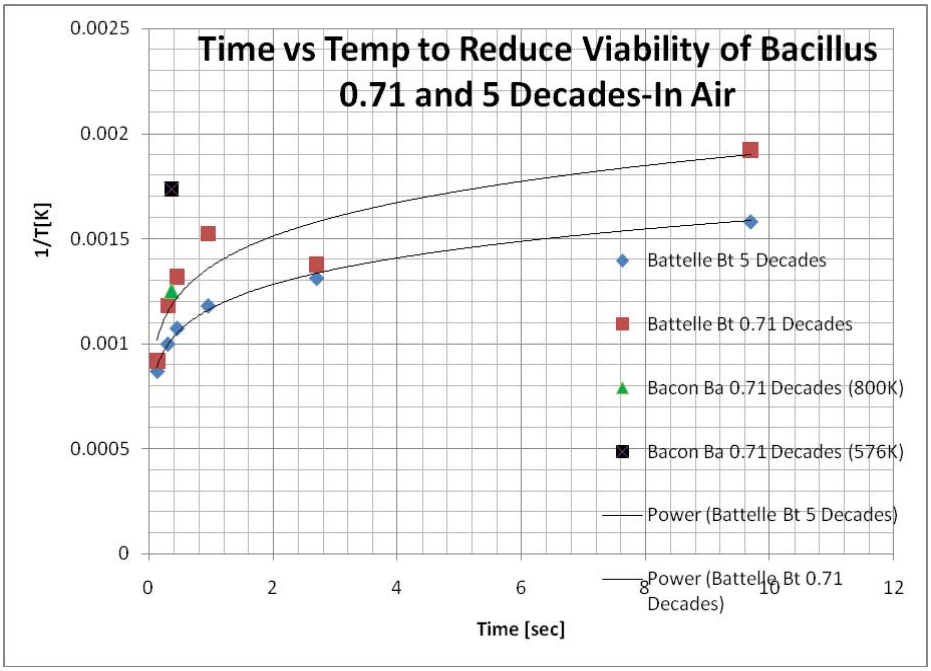


Figure 22. Bacon (*Ba*) versus Battelle (*Bt*) Data at 0.71 Decades

Given that the data point falls atop the trend line for the 0.71 *Bt* reduction, points towards a validation of the methodology compared to Battelle's.

The second reason that comparison of data is difficult has to do with the heating method used in this study. The heating method used treated the samples at a constant raster rate, but at three or six exposures to achieve longer exposures because of current raster rate limitations. The treatment duration, was therefore the product of the number of exposures multiplied by the duration of one raster over the 50µm puck; see Table 6. The assumption made to support this assessment, based on model calculations by Dr Baker and Capt Emily Knight, is that as soon as the micro-black body is struck by the laser the entire well begins to see the treatment temperature. At the slowest raster rate, the longest single exposure per well that is able to be achieved by this experimental protocol is 0.85sec. If the duration of exposure is controlled not by rastering, but as a function of increasing and decreasing the laser power then a point treatment of individual wells can theoretically be achieved at an infinite number of durations. The Battelle© data uses a single heating exposure; one long burst compared to three/six shorter bursts. Figure 22 compares the Battelle kill probabilities with this work's at a constant temperature of 576K.

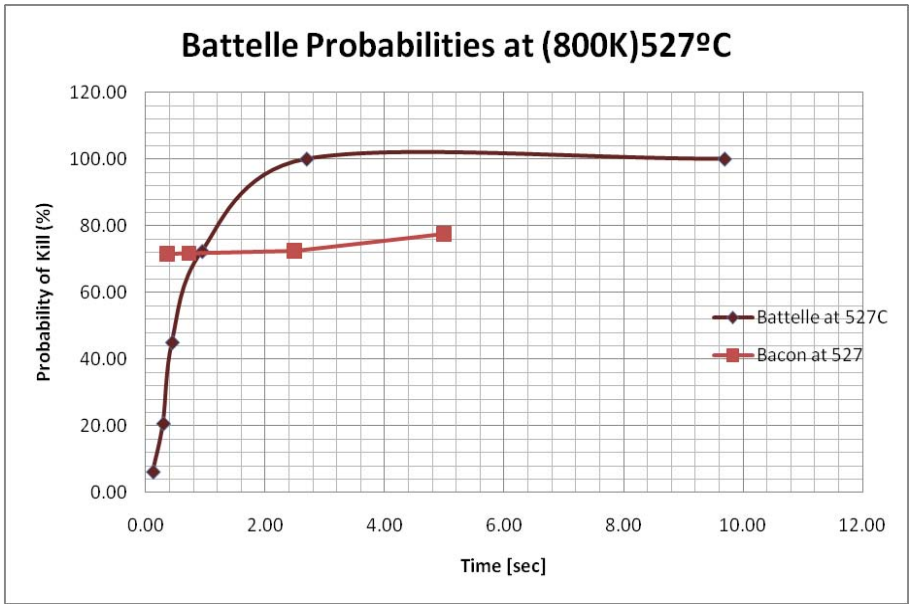


Figure 23. Time vs Probability of Kill at a constant temp 800K (527ºC)

4.4 Investigative Questions Answered

RO1: Address previously identified problem of measuring both spore growth and spore thermal kill threshold using the traditional method of spreading a diluted spore solution on a plate.

Though the data produced in this work failed to provide any further insight in to *Ba's* thermal kill threshold, it did further refine the preparation and population of an etched platform with a diluted spore solution shown to be useful to irradiate spores and to measure delay in germination. Previous work focused on saturating 5-10µm wells with a large density of spores. The idea behind this protocol was that a large amount of spore density was required to overcome the hydrophobicity and hydrostatic tension challenges associated with populating a hole of that miniscule size. One of the primary challenges associated with that method was difficulty in cleaning excess spore deposition from the

surrounding surfaces outside of the wells. Tremendous effort was spent trying to repeat this protocol. Common problems included damage/destruction of the comparatively frail platforms. Also, it is arguable that qualification of the wells as populated or unpopulated was very subjective and by no means definitive. Growth runs to validate the population protocol were conducted by Hawkins, however, the same qualification could not be done for the actual samples being treated (Hawkins, 2008). The effort to quantify the previous population technique was based on outgrowth, but for inactivation trials the criteria was only populated or not populated based on a subjective shading of each well. Populated wells could, theoretically, contain one or many spores; it was not definitively determined because the well diffracted the light making it impossible to image individual spores. An argument could be made regarding the maximum number of spores per well based on well volume, but the fact remains the quantity per well was estimated at best. During early efforts to reproduce Hawkins' protocol, a modification using fluorescent microscopy was made in hopes of better qualifying the wells. The use of fluorescence did not provide that much benefit in terms of microscopy. It did, however, lead to concerns regarding the impacts on the spores' susceptibility following staining and observation under UV conditions. To mitigate these concerns, the staining was executed using a less invasive protocol, cold staining with no heat fixing, and the UV used during microscopy was low power. It was determined that the method could be used in validating the biological population protocol, but it could not be used to quantify samples for use in the thermal runs. The benefits of the technique did not outweigh the effort and toxicity concerns. The population and cleaning technique developed by Hawkins proved

very difficult to reproduce. Using the protocol outlined in this work, it was possible to qualify and quantify the wells following population. The actual number of spores per well for the 50 micron wells was determinable using microscopy. Microscopy techniques allowed for the counting of individual spores before and after treatment. Photo documentation of well populations was accomplished before and after growth allowing the researcher to compare and determine viability after three hours of incubation. Though observation through the backside of the coverglass offered a different level of clarity and the before/after images were superimposed, it was possible to assess the viability of individual spores through direct comparisons and applicable vegetative growth. Wells were identified by an alphanumeric naming convention and able to be located time and time again. No attempt at assessing growth rates were made, however, similar methodology to Hawkins was used for defining separate genetic lines to prevent double counting and false negatives.

RO2: Refine production of micro-etched platforms to facilitate spore separation. Intent is to prevent neighbor growth from obscuring germination and initial vegetative growth measurements.

The production of micro-etched platforms, from both coverglass and microscope slides, was instrumental in the work. Two custom photomasks were designed in support of this effort. Both masks were designed to facilitate batching in an effort to increase output and minimize production time of sample platforms. Additionally, well spacing and well diameter were changed to better address thermal diffusion concerns.

Clean room protocol. No major changes were made to the clean room protocol with the exception of batching efforts and equipment used during exposure. All sequences and wet chemistry processes remained unchanged from Hawkins. Batching efforts included the use of slide holder and wafer baskets to treat five slides versus just one during the development and etching steps. The one major difference in the process was in the exposure of the platforms where the EVG620 Mask Aligner® was incorporated in to the work. The EVG620 allowed repeatable exposures, reduced exposure time, and improved precision compared to previous protocol using the Karl Suss MEMs mask aligner. The mask and samples can be aligned exactly each time using platform guides and controls machined to a high precision. The etching process remains a variable. Inconsistencies in the starting substrates, glass coverslips and slides, became obvious during microscopy observations. These same inconsistencies are the cause of variance in the quality of etching. Additionally, the strength of the hydrofluoric acid decreases over time and following multiple uses. This degradation is not well characterized with a calibration curve for example. As a result, an etched platform produced during an early run is different from that of a later run, respectively, if the same BOE is used. During large scale production the BOE was refreshed after the processing of 30 platforms, but what variance this caused remains undetermined.

Well Size. Early efforts focused on producing a biological platform with 5um wells. Intent was to populate each well with as few spores as possible, treating each well as a sample. As difficulties were encountered in the population and measurement of these smaller wells, the decision to use the 50µm wells for biological population was

made. This further improved the possibility of black body/biological sample alignment during final configuration given the fact that the same mask was used to produce both etched platforms. A well size of 50µm was incorporated in to the final protocol out of necessity; did not have time required for the design and production of another mask. This much larger well size was much easier to populate than the 5µm wells. An analogy to describe the difference using the larger well made would be to compare it to throwing a basketball into a swimming pool (50µm well) vice a standard basketball hoop (5µm well). The 50µm well was actually too large and added uncertainty in to the thermal model; increased air gap surrounding spores. A well diameter of 25µm would be ideal to balance the ease of qualification with thermal modeling.

Spacing. The decision to increase the spacing between wells, from 250 to 1500µm, was made to mitigate thermal diffusion concerns across the glass medium. Concern was that neighboring wells would be heated prematurely by lateral diffusion as nearby wells/rows were treated. Modeling efforts by CPT Knight (Knight, 2009) mitigated much of this concern showing that at the target temperatures and durations very little lateral thermal diffusion is seen. The larger spacing, however, was both harmful and beneficial to the effort. During the actual thermal treatment the larger spacing was a benefit in that a smaller spacing would have made the alignment and rastering technique more challenging. The spacing was just large enough to observe a noted difference between when the laser was atop a well and when it was not, by the light diffraction pattern. The larger spacing made microscopy efforts more difficult during biological population of the platforms using the 5µm wells. If significant debris was present outside

of the wells, identifying the characteristic row/column pattern of the etched platform was near impossible at lower magnifications. At 100x magnification, the 50µm wells and the corresponding patters is readily identified. Using the 5µm wells, at this larger spacing, a magnification of 400x is required. This much smaller objective view makes rastering rows and columns much more difficult. Finally, the larger spacing also reduced the potential sample size. As a result, a larger coverglass was selected for the starting substrate in order to increase sample size; 22x60x0.17mm versus the previously used 22x22x0.17mm.

RO3: Refine characterization of the thermal heating environment. Model sample configuration to determine heat loss between source temperature and that which the spores are exposed to.

The larger diameter wells were designed, as indicated, for the production of micro-black body irradiators. These irradiators were designed to replace the previous black body irradiator used in Goetz' work which was SiC sandpaper. The SiC sandpaper used in the previous work was a sheet backing compressed against the biological sample. A concern with the use of SiC, is that the irregular surface would not absorb, emit and scatter thermal radiation in a uniform manner and the conductive heating produced two treatment temperatures. Other materials such as carbon foil or pyrolytic carbon were analyzed for use, but none of the choices lost heat quick enough nor were they reusable. Costs as a function of durability ruled out the use of carbon foil. Additionally, in order to achieve a thermal pulse with well characterized ramp up and loss of heat, (leading and trailing thermal tails), something other than a sheet was needed. The concept of a black

body microdeposit, or ‘puck’ was discussed and deemed ideal for the heating configuration. The diameter of this micro black body is greater than that of the biological sample in order to facilitate some margin of misalignment error. Additionally, alignment features were added to the etched platform design in order to facilitate the macro and micro alignment of the two samples; biological with black body. Failed to characterize wells; depth and air presence. Failed to characterize final sample configuration; specifically air gap between bio and black body platforms.

Early criticisms of the work associated with heating a biological platform by means of a black body irradiator was that the temperature at which the spore was exposed was something less than the source temperature. The thermal model outlined in paragraph ‘2.5.2 Thermal Diffusion’ attempts to model the thermal properties of the sample configuration. The thermal conductivity of glass and air is used to calculate the temperature at the surface of the black body irradiator. This temperature is a percentage of the source temperature and most affected by variance in the air gap between the biological sample and the black body irradiator. Poor air conductivity accounts for the greatest difference in temperature. Assumptions are that convective flow and radiant heating are negligible. The model does not account for exposure time and assumes a steady state for the model given the fact that thermal diffusion is rapid compared with times of thermal pulses associated with conventional munitions; (Baker & Burggraf, 2008).

RO4: Determine the effects of environmental conditions, ie: desiccation, humidity, sporulation temperature, ambient temperature (cold shock) on the spores' susceptibility to thermal inactivation strategies.

This research objective was not met. Early biological population trials were conducted with thorough documentation of sporulation temperature and desiccation following population of the biological samples. Those trials were not used in the actual data collection of the work. Conditions of spore formation alter resistance to wet heat, toxic chemicals and sterilization; specifically water and minerals. The extent of desiccation, does not however impact susceptibility to dry heat and UV radiation, (Baker & Burggraf, 2008). Sporulation conditions were constant for data runs therefore no data was available to speak to impacts of sporulation temperature on susceptibility to thermal inactivation.

RO5: Assess the inactivation strategy's ability to achieve a 6-Log reduction; 99.9999% probability of kill.

The inactivation strategy is more subtle than many of the efforts conducted to date. As a result it lacks the sample size to ever meet a 6 Log standard. Complementary strategies, compatible with the laser heating strategy using larger sample sizes must be developed to achieve this standard. Most strategies measure effectiveness of the respective strategies in terms of Log reduction because starting population densities on the order of 10^5 thru 10^9 are used. The subsequent reduction, down from 10^9 to 10^7, would then be characterized as a 2 Log reduction. This protocol does start with a population density of 10^9, however, the population technique is specifically designed to

reduce that density to a manageable number of spores per platform. In essence, the starting population density per biological sample platform is in the hundreds not thousands; see Figure 18 which shows mean spores per well by platform. Comparison between the two types of data is difficult if not impossible. Theoretically, it is possible for this protocol to generate a data set capable of testing effectiveness to the 6 Log reduction standard. It would, however, require thousands of sample platforms and researchers. It is arguable that the quality of this data is better, given precision in counting. The protocol does not rely on culture plate counts which speak in terms of colony forming units. The determination of a colony forming unit is gray at best; overgrowth of one spore's vegetative cells atop another could result in overestimating the strategy's effectiveness. This protocol looks at individual spores and assesses each spore's response to the strategy in question. As a result, this increased precision in assessment of germination is balanced by a decrease in sample size. This microscopic counting method, even in an automated form, is best suited to measure germination delay following thermal treatment for spore populations of several hundred spores, permitting only a 99% log kill determination.

***RO6: Further assess the use of* Bt *as a comparatively safe surrogate organism for* Ba.**

Bt was the primary organism used during early population trials. During data collection it was not used due to questions regarding stock sample production. The sporulation protocol used to produce the *Bt* samples, provided by Brooks AFB, were not known. It was also determined that both samples included additives which changed the

physical characteristics for comparison with *Ba*. In the case of *Bt (Toast)* flow enhancers, believed to be a silicone based additive, were added to better simulate weaponization. For *Bt (Javelin)*, the commercially produced stock included surfactants to prevent clumping and ease distribution for agricultural applications. Both stock samples, required a modification to the washing/concentration steps of sample production. During the second wash/centrifuge cycle a diluted ethanol solution was used to remove the silicone based surfactant or flow enhancer. This chemical treatment raised concerns regarding what impact it would have on the organisms' susceptibility to thermal inactivation. The decision to remove the organism from the study was made. Either of these two *Bt* variants, if used merely as a stock to inoculate and grow cultures in house, could have been used to compare against the *Ba* samples used in the study. Time was the main reason this objective was not met.

5. Conclusions and Recommendations

The primary goal of this work began as a pursuit of larger, statistically meaningful data sets using an established protocol. The experimental design of the etched microscope platforms and materials procured would have facilitated the achievement of this goal, however, sample preparation proved challenging and time consuming. A shift in priorities occurred resulting in the development of finding a reproducible protocol for producing microscopic platforms and demonstrating their utility in spore inactivation as this work's primary objectives.

5.1 Conclusions of Research

A method was developed for production, population, characterization and inactivation of *Bacillus anthracis* using a micro-etched platform for separation of spore samples. Improvements in the population technique were realized as a result of changes to well size, spacing and alignment features for the micro-etched platform. The primary conclusion derived from the biological population trials is that a more dilute, lower population density, sample is most effective. Lower density samples mitigate many of the production challenges encountered by the previous efforts; specifically cleaning excess spores depositions from the surrounding glass surface. At the population densities used in this study, overgrowth of vegetative cells did not prove a concern during post treatment assessments. If multiple counts, however, are to be completed as part of future efforts a lesser dilution should be used. A smaller dilution will reduce the amount of unintentional deposition outside of the sample wells, therefore, improving the precision

during counting. This will reduce the number of wells per platform that are populated with a viable number of spores, but the quality of data per treated well increases.

The forces most responsible for the successful population of the biological wells were gravitation, surface tension and evaporation. At the microscopic level, overcoming the wells' surface tension through shearing or compressive forces is too blunt of a method for a subtle process. Spores suspended in water constitute the sample. Spores either deposit quickly, through gravitational forces, or they remain suspended in the water layer until evaporative forces pull them downward and deposit them on the glass surface. The lowest points evaporate last which is why the spores appear to be pulled in to nearby wells during drying. Finally, the ideal well size for the production of a biological sample is 25µm in diameter. With the current design, spacing of the wells proved too great for reliable microscopy during population trials using the 5µm wells. Additionally, qualification of the 5µm wells as populated or unpopulated is subjective and technique based. Trials were conducted using fluorescent microscopy techniques, but these provide little benefit when viewing a concentrated spore sample.

Fluorescence, cannot be used to characterize samples for use during inactivation runs due to toxicity concerns associated with UV irradiation and the staining process. The quantification of 5µm wells, before and after treatment, is impossible unless scanning electron microscopy (SEM) is available. For the production of large data sets, the use of the SEM is not efficient or cost effective. A 50µm is large enough that population is comparatively easy to qualify and quantify. The total volume of the well, however, is too large in that it introduces too much air in to the sample system. Increased

air surrounding the treated spores results in heat loss and reduces the thermal characterization of the treatment strategy.

Improved thermal characterization of sample configuration and heating method was achieved. A micro-deposit of carbon black was polymerized using a wet chemical technique referred to as a sol-gel process. At the slowest raster rate, the longest single exposure per well that is able to be achieved by this experimental protocol is 0.85sec. Fastest single exposure using this protocol is 0.12sec. If the duration of exposure is controlled not by rastering, but as a function of increasing and decreasing the laser power than a point treatment of individual wells can be achieved at longer durations.

The micro-black body was developed to replace a backing of silicon carbide, (ie: sandpaper), which was used in earlier work to function as the macro-black body irradiator. The carbon black improved heat loss due to reflectance caused by the irregular pattern of the silicon carbide sheet and improved the irradiation profile. Improved stability of the carbon black deposit was achieved by a sol-gel treatment. However, final black body well configurations were insufficient to withstand laser settings equal to or greater than 1.6W. Though not all wells were destroyed, a significant number were observed.

The novel capability to use laser irradiation of micro-etched black body radiators to inactivate spores was demonstrated.

5.2 Recommendations for Future Research

Etched Platform Production. Though improvements were made to increase the throughput of and reproducibility of etched platforms, quality control can further be

improved. A significant variance between batches and even wells on the same platform was observed over the course of the study. Well quality concerns as a result of this variance impacted population efforts; especially during carbon population. Contributing to this variance may have been the quality of glass used as starting substrates; soda lime coverslips and microscope slides. Using a higher quality glass, or quartz, may facilitate a more uniform etching. Substrate alternatives could facilitate an improvement in well quality, sharpen well slopes and the reduce lateral damage caused by the etching process. Recommend working with clean room technicians to improve the quality of etched wells and better characterize etching rate of soda lime glass to reduce variance. Many of the wet chemistry techniques used are not designed for the large scale of production associated with this work. Protocol adjustments to refine photoresist selection, etching concentration and subsequently etching rates may be in order. The thermal model used to characterize the heating environment, associated with this sample configuration, would benefit from a good characterization of the etched platforms used. Characterization of etched platforms can be accomplished using the SEM and profilometer.

Biological Sample Preparation and Assessment. Future efforts should attempt to populate a 25µm etched well. More dilute samples should be used and efforts to improve the excess spore cleaning step during population. The population density used was not so large that post treatment assessment was impacted. If additional counts are executed, (i.e.: 3, 6, 9hr), then even the smallest amount of spore debris nearby sample wells could impact viability counts. Sacrifice the number of wells populated for a lesser number populated very well.

Execute multiple counts, following timed incubations, to ensure spores classified as nonviable at the 3hr mark remain nonviable hours later. This will mitigate concerns associated with false positives associated with spores that have been damaged, but not killed.

Applications for Biological Platform. Using the biological sample production protocol outlined in this work, experiment with alternative inactivation strategies; specifically attempt wet heat, chemical, and ionizing radiation. These methods may not be feasible to test individual wells or rows with the precision of a laser, but a valid sample set could be derived for platforms treated completely by the inactivation strategy of interest.

Applications for Carbon Black Body Platform. If a technique could be developed to micro deposit biological samples in the same configuration and pattern as the black body irradiator, an alternative sample configuration would be feasible. The current heating technique using the solid state laser could be sustained, but the question of thermal characterization would be answered. One of the concerns with the current methodology is that the spores do not come in direct contact with the irradiator or that the air gap between spore and black body is too great. Under computer control, an ink jet-like tool could be used to microdeposit spore slurries atop of the carbon wells. Before and after treatment counts would have to occur using a top illuminated microscope versus phase contrast.

Thermal Inactivation-Dry. The sample configuration designed for this work was effective, however, it is flawed. The compression of the two biological sample

platform and the black body platform is poorly designed. The alignment necessary to ensure that each biological well is in contact with a corresponding black body well is impossible to ensure for all samples. The tolerance /error built in to the photomask is too great to produce mirror image etched platforms at different well diameters. Even using the same mask to produce both biological and black body platforms is not possible due to fact that orienting wells with working surfaces facing one another would not create a mirror image for more than one row. Fewer wells could reduce this propagation of errors, but would reduce sample size. If this two layer sample configuration is used again, longer duration trials are possible even though the slowest raster was used in the aforementioned data sets. Current protocol calls for multiple exposures using a constant raster rate. Long duration, single pulses, can be achieved by centering the laser atop individual wells and cycling the laser power from its calibration power, 0.2W, to the target power to be used for treatment. The transition is computer controlled and occurs in less than a second, but strict timing could account for this fact.

5.3 Significance of Research

Ultimately this work established a technique to separate, heat and assess biological spores before and after treatment. The original intent of using etched platforms was to enable separation between spore sample allowing researchers to observe/assess without the background noise/confusion of abundant germination; encroaching neighbors. Early methods populated small wells, 5-10μm, with as many spores as possible per well. This work utilized larger wells with less spores; on average 4-10 per well. These refinements improved population qualification and quantification,

ultimately improving the post treatment assessment of kill probability per set of trial conditions.

Unique to this work, is the production of a custom black body irradiator which allows for a sharp thermal pulse without the concerns of characterizing the tails associated with the thermal heating profile. The black body production included a stabilization step utilizing a sol-gel application which simultaneously binds with the platform's glass surface and the carbon wells. Overcoming the challenge of coating two mediums, one partially hydrophilic and one partially hydrophobic, was done through experimental trials using different rates of spin coating with different dilutions of organosilane. Though the laser heating method using the FT-Raman is not unique to this work, it has never been applied to heat a row of 50µm sample wells. At this point, individual wells and spores can be isolated, thermally treated and assessed per well in a repeatable method. The precision at which the wells can be located and then treated facilitates the opportunity for a near limitless range of time/temperature inactivation trials. Limiting factors would be the laser power and minimum duration of 0.16sec. Treatments can be conducted in batches, (using rows), or can be point treated, (individual wells). Regardless of sampling technique used, the etched platform provides opportunity to produce a variety of accurate data sets all on the same platform. This development will enable future work relevant to several inactivation strategies to include dry heat, wet heat, chemical and radiological.

Appendix A. *Bacillus anthracis/thuringiensis* Sporulation Protocol

Citation: Protocol provided by Ms. Angelica I. Rubio, CB Senior Scientist, 709th NSS, Kirtland AFB, NM.

Purpose**:** The method is used to ensure a high production of spores synchronizes the *Bacilli* cell growth so that when starvation is attained and quorum signals are sent, the highest possible number of cells are ready and capable of sporulating. Once sporulation is complete, the basic approach is to spin down all the material in a culture. At this point, the percentage of spores, or cells with spores in them, is usually 90%-98%. Sporulation in the *Bacilli* is a one to one relationship; one mother cell yields one spore. In case there are spores trapped in mother cells, the last stage of the culturing is done in the presence of water. This helps to release most, if not all, of the spores.

Materials and Reagents:

- PA Media:
  - $CaCl_2.2H_2O$ 0.15 g/L
  - $MgSO_4 \cdot 7\ H_2O$ 0.2
  - $MnSO_4 \cdot\ H_2O$ 0.05
  - NaCl 5.0
  - Nutrient Broth 8.0
  - Double Distilled H_2O
  - Bring pH to 6.0 with HCl
  - Aliquot 100mL/125mL bottles

- Blood Agar plates (#01-200, Remel, Lenexa, KS)
- 500-ml orange-capped sterile flasks
- Sterile Oakridge tubes

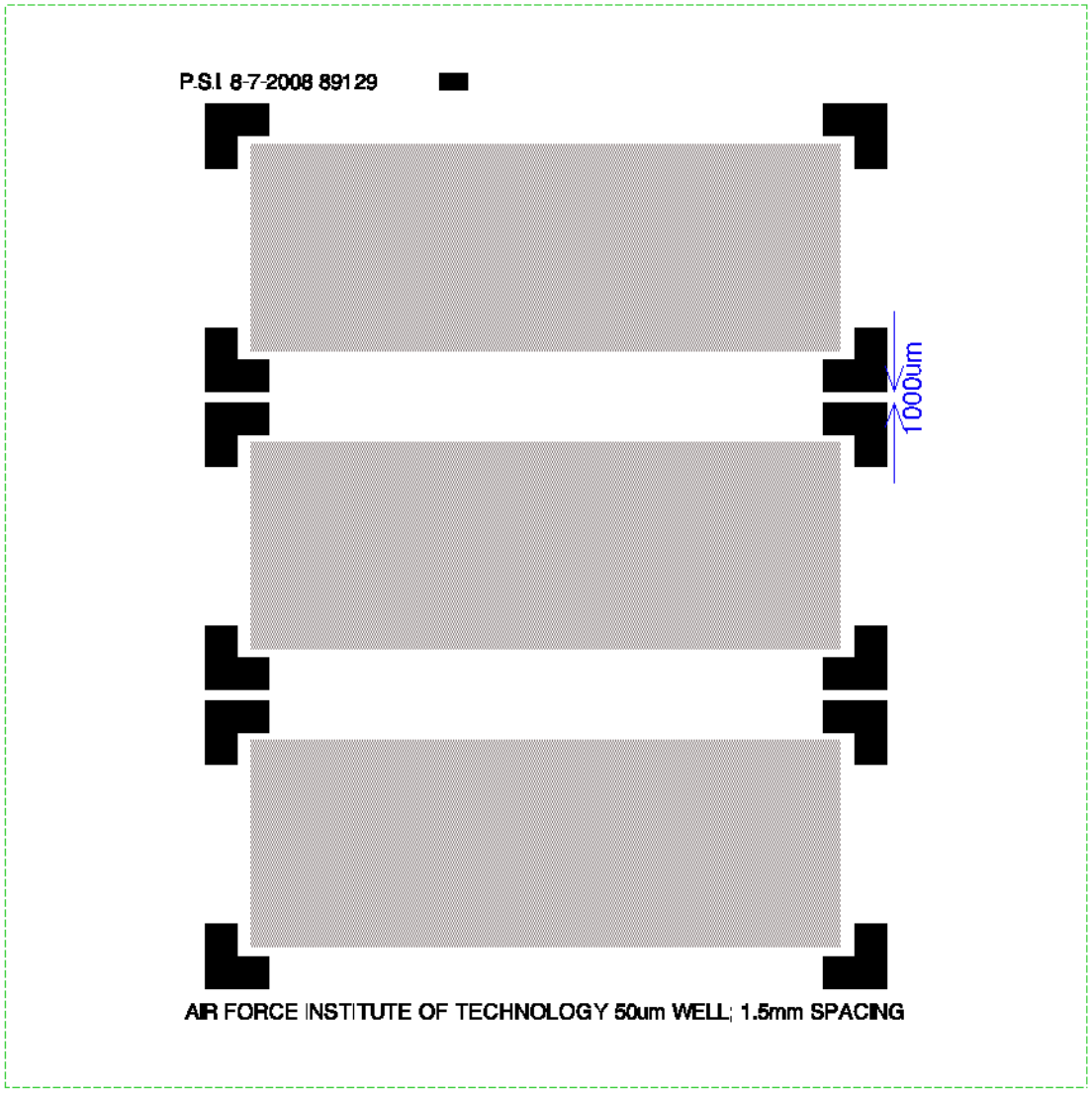
- Cold sterile dd H_2O
- Sterile Inoculating loops
- SS-34 rotor, Sorvall superspeed centrifuge
- Culture shaker
- Incubator
- Sterile, screw-top epindorph tubes
- Select Agent Incubator
- Automated Spiral Plater (Spiral Biotech)
- Scanner for cfu determination and quantitation (Spiral Biotech)

Procedure:

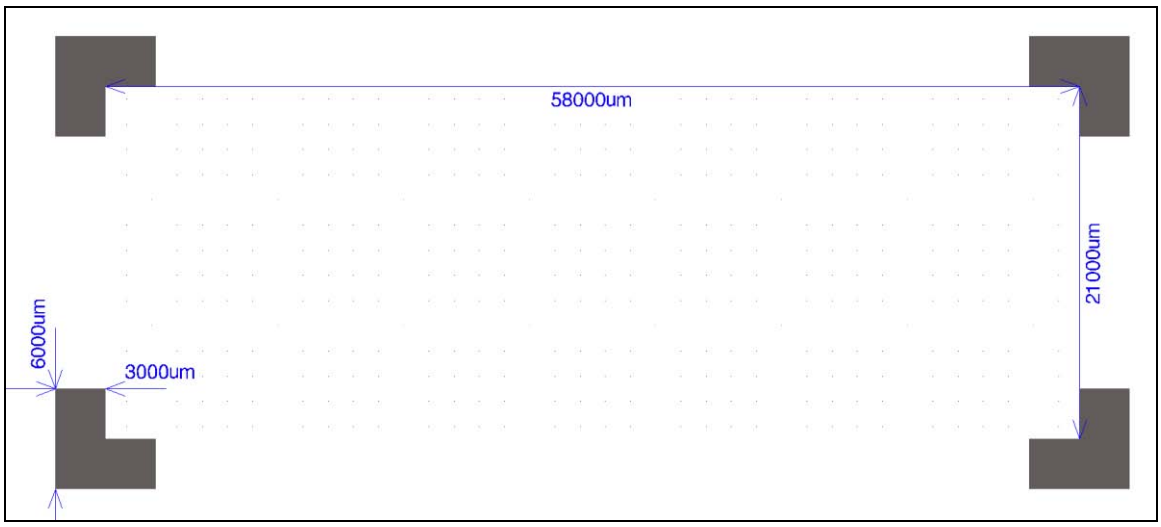
- Growth Activation (Note: Requires two people).
    - Remove tube of *Bacillus anthracis/thuringiensis* original master stock from Select Agent –80 ^{o}C Freezer, and allow to thaw.
    - Using a sterile inoculating loop, streak 10 ul onto a blood agar plate.
    - Incubate overnight at 27 ^{o}C
- Preculture
    - Inoculate pre-warmed 25 ml PA in the 500-ml orange-capped flask, with one colony from the overnight plate culture.
    - Shake for 24 hours at 37^{o} C at 350 rpm

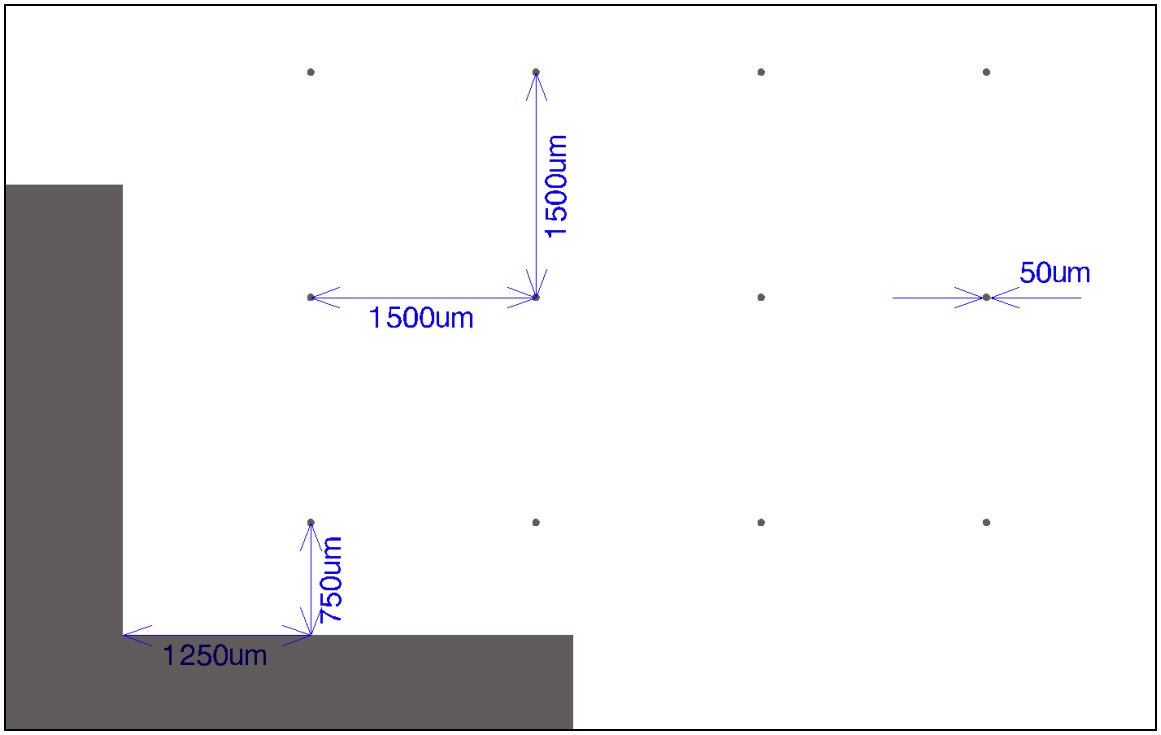
- Sporulation culture
    - Add 1.25 ml of the preculture to 50 ml of PA (pre-warmed) in a 500-ml filtered-top, plastic flask and shake for 24 hours at 37^{o} C at 350 rpm
    - At the end of the 24 hour period, add 200 ml of sterile dd H_2O and continue shaking at 350 rpm, 37 ^{o} C for 40 more hours.
- Spore harvest
    - After the 40 hour incubation check for complete sporulation. Place 1 ul of spore prep on glass slide.
    - Cover with coverslip.
    - View at 100X on phase contrast microscope. Spores should be phase bright. If spores are not apparent, incubate culture longer – checking for phase bright spores every two hours.
    - Transfer culture to a sterile Oakridge tube and spin in a Sorvall superspeed centrifuge using a SS-34 rotor at 4000 rpm, 4-10^{o} C, for 20 min.
    - Discard the supernatant wash the pellets with sterile PBS.
    - Resuspend pellets in 50 mls of sterile PBS.
    - With a partner, aliquot 500 ul per sterile, screwtop vial and freeze at – 80 ^{o} C. If applicable, make select agent tracking form with appropriate number of vials. Both individuals involved in the aliquoting should sign off on the tracking form.

Appendix B. Photomask Design



(Appendix B. Photomask Design Continued)





Measurements are from center of circle to center of circle
Opaque areas on plot to be clear (soda lime) with white areas to be chrome at mask
Array of 50um circles: 38 columns x 14 rows
Total: 532

Appendix C. Statistics

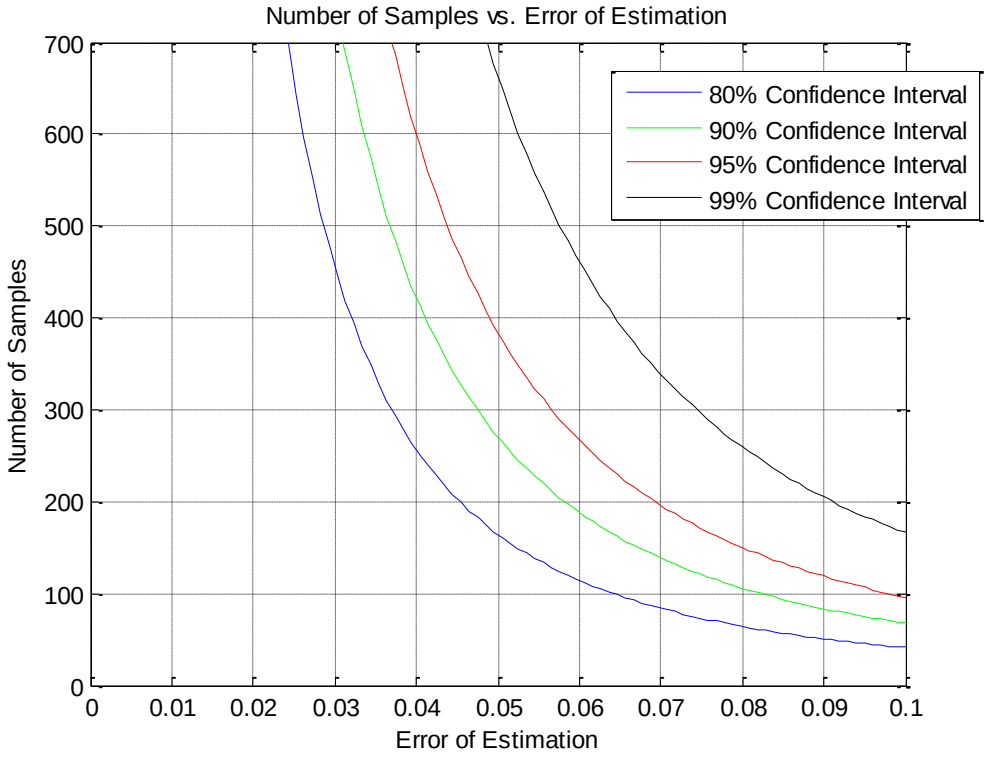
Formula to determine number of samples needed:

$n=(z^2pq)/(b^2)$

n=number needed to sample
z=confidence interval based on the standard normal distribution (ex. z=1.645 for a 90% confidence level)
p=probability of success (since we don't know the probability of success of our population, we have to assume p=.5)
q=probability of failure (1-p)
b=error of estimation (ie, difference between the estimator and its target parameter)

Below is the graphical representation of the number of samples that need to be taken based on different confidence levels and errors of estimation.

(Wackerly, Mendenhall, & Schaeffer, 2002)



Appendix D. Lessons Learned Black Body Production

Problem Defined

Populate 532 wells (5um in diameter) per etched coverslip with particulate carbon black; ≥85% of the well.
Once populated, remove excess carbon debris from the surface of the etched platform without depopulating the wells.
Stabilize the carbon within the wells to facilitate repeated experimental runs.

Flawed Slides

-*Problem*: Wells too shallow; unknown depth. Variance due to etching rates as hydrofluoric acid (BOE) looses strength over the course of batches/etching.
-*Control*: Confirm depth in clean room using profilometer or scanning electron microscope (SEM). Note: This step was not executed during production and experimental runs. Some slides/wells just never populated regardless of repeated applications; wells were just too shallow or malformed.

-*Problem*: Wells are obligated by water or advantageous hydrocarbons from ambient air.
-*Control*: Various cleaning methods were used to clean and dewater slides prior to the carbon application; methanol, touluene, ethanol and glassware soap. Chemical treatments alone where insufficient to prepare the slides. Sonication of slides in ethanol serves to 'degrease' the slides by removing debris and by removing any hydrocarbons that have bonded to the wells' surface. The slides are emerged in water following sonication to obligate the bonds with water molecules, therefore, preventing any opportunistic hydrocarbons from bonding to the wells during transfer to the oven from ambient air. The heating of the slides at 150°C for 15 minutes serves to drive off these water molecules leaving a well ready for carbon population. Dessication during cooling is less a function of drying and more of prevention. If allowed to cool at ambient conditions the slides would see an uptake of water molecules while cooling in the dessicator prevents this. Finally, the carbon/silane application occurs within an N_2 environment for the same reason.

Application Technique

-*Problem*: Vertical/shearing pressures are not uniform on the material and wells are left unpopulated.
-*Control*: Application tools experimentally tested included a Teflon© policeman, glass microscope slide, manufactured edge of a weigh boat, and finally a custom machined Teflon© block. Partial population of wells was seen throughout trials; quarter moon, crescent moon and donut patterns were all observed. Observations showed that there was irregular, possibly insufficient, downward and shearing pressures being asserted against the material during placement. It was also surmised that depopulation was occurring during the population process; especially during secondary and tertiary applications. The

(Appendix D. Lessons Learned Black Body Production Continued)

The glass slide trials were intended to create a uniform downward pressure. Shortcoming of this method was gauging of the black body slide and depopulation due to equivalent materials; carbon was as prone to stick to applicator slide as it was to black body slide wells. Early runs with the Teflon© policeman met the desired characteristics of a non-stick surface, but lacked the uniformity and surface area necessary for a uniform downward pressure. The final application technique, the Teflon© block, met both of the required characteristics—large, non-stick surface area capable of the necessary uniform, downward pressure. If carbon was moist or had even been exposed to ambient lab conditions the carbon tended to clump or stick to the Teflon© block.

-*Problem*: Particle separation due to physical properties of carbon lamp black; finer (more desirable) particles were removed by the fume hood and air currents.
-*Control*: A glove bag helped prevent the carbon black from separating due to air flow and/or from clumping due to relative humidity in ambient lab conditions.

-*Problem*: Static charge created during application and scraping/cleaing pulled particles away from target slide.
-*Control*: Ground each slide using copper wire and gator clips. Alternative method, utilized during research, was a methanol swipe of the scraping/cleaning tool between every use.

Slide Cleaning/Reuse (Post Application)
Problem: Once organosilane is added, at any concentration, slides are no longer useable.
Control: Using a 1M NaOH solution tried cleaning failed slides at durations ranging from 1minutes to 5hrs. Trials to date have determined that as short as 5 minutes is too short while 5hrs is too long; pitting occurred while organosilane remined in place. Sonication of slides bathed in ethanol cleaned dry application slides, however, failed to do anything except remove surface debris from slides with paste or slurry applications.

Appendix E. Lessons Learned Biological Sample Preparation and Population

Problem Defined

- Populate 532 wells (5um in diameter) per etched coverslip with one or more *Bt* spores.
- Once populated, remove all excess organic material from the surface of the etched platform without depopulating the wells.
- Protocol cannot change spore's susceptibility to inactivation strategy of interest.

Summary of Observations to Date

- Population of wells unsuccessful
- Confirmation of population indeterminate
- ECS configuration less than ideal; microscopy challenges
- Well spacing; 1.5mm
- Well size; 5um diameter small
- Debris confusion
- Cleaning protocol in question
- Detergent leaves residue
- EtOH leaves a cleaner ECS
- Fluorescence aids microscopy, but may inhibit spore
- Standard Schaefer-Fulton protocol unusable; heating and washing
- Cold staining stains everything
- Toxicity of stain and UV treatment in question
- Scraping/Excess Spore Removal
- Cannot replicate Hawkins protocol; presence of debris

Experimental Parameters/Protocols

Sample

1. Well characterized sample? No agar, no vegetative debris, but has debris…..
    a. Bt (kurstaki)
        i. 'Toast'; from Dugway with flow enhancers--silicone
        ii. 'Javelin'; off the shelf biopesticide from Certis
            1. (85%Bt; 15%'Other Ingredients'-surfactant and sodium sulfate)
2. Limited quantities
    a. Loss of sample
        i. Size of satellite vessels, conical test tubes, etc
        ii. Material removed as excess

Etched Coverslip (ECS) Production

- Cleanroom protocol for production unchanged with exception of EVG620 and batching efforts
- Configuration variations

(Appendix E. Lessons Learned Biological Sample Preparation & Population Continued)

- Well size larger; 5um vs 3um
- Spacing increased; 1.5mm
- Larger coverslip; length is greater but no known material differences-sodalime glass

ECS Cleaning. Prepare ECS for population with *Bt* by removing all debris from surface of ECS and inside of wells.

Detergent Protocol

- Residue remained prior to population; causes micro challenges post pop and scraping
- Benefits of detergent pertinent to surface tension?
- Only difference from Hawkins was batching?

EtOH sonication protocol

- Developed during Black Body production
- Mechanical swab, EtOH sonication, soft bake, cool in dessicator
- Differences from carbon app
- Wet vs dry population
- N_2 environment
- Less residue than detergent
- Soot from oven; opportunistic hydrocarbons?

Bt Population. Deposit one or more spores in every well.

Spore Prep

Washing?

Slurry

70% EtOH –vs- H_2O

EtOH

- Intended to overcome challenges associated with hydrophobicity of spores
- Compliments EtOH/Sonication cleaning method developed during carbon production
- Kirtland protocol using EtOH to rehydrate
- Used by Kirtland to remove silicone (flow enhancers) with 'no evidence of spore coat damage… EtOH is for silicone removal, and the period of time will not affect the spores'
- 1:1(mg/ml) ratio; Note: proved too wet!
- EtOH toxicity
- Retarded growth rate at 5, 12, and 24hrs.
- Early runs with w/minimal contact time at 70%. Perhaps a lesser concentration?

H_2O

- Compliment Detergent rinse

(Appendix E. Lessons Learned Biological Sample Preparation and Population Continued)

- Proven protocol; technique dependent
- pH; adjustment in pH 'impacts on ion-dipole intermolecular forces between the ammonium ion and the silanol groups'

Staining

- UV toxicity; ref Whitney et al
- Post thermal treatment applications only?
- Pre treatment to determine spore distribution and protocol certification
- ID row/column pattern using 200xPC; switch over to UV to validate well pop. If green hue present, success—speak to depth of vision observations
- Impacts to microscopy; good and bad.
- Blue hue; debris—observed on both populated and unpopulated ECS
- Green hue, spores—just spores?
- Impacts to population success; physical properties of slurry?
- Cold staining
- Physical properties of sample and interaction with glass surface?

Excess Sample Removal. Remove all surface debris that could inhibit an accurate cell count post treatment and incubation without depopulating the wells.

- Cell scraper, weigh boat edge, weigh paper
- Methanol swab
- Edges and back of ECS

Bt *Population*

- Dry Population—wasteful?
- Adjust Slurry
- EtOH; <70%
- Phosphate *Buffered* Saline (PBS)
- "As far as the material going into solution. We use a 1mg/ml ratio diluted in PBS + 0.1%SDS for the old stock. So for 500mls they add 5mls of 10%SDS. The sample is vortexed vigorously and easily goes into solution. Pedro says he gets about 10^8 cfu/mg. For the new stock, still 1mg/ml ratio, but sample is diluted in 70% EtOH. This goes into solution easily, once vortexed. It is titered in the same PBS+ 0.1%SDS. The count for this stock is about 10^5cfu/mg."
- SDS Solution (10% w/v) is sodium dodecyl sulphate in distilled, deionized water. SDS is a detergent that is known to denature proteins…?
- Excess Removal
- Rinse and wipe technique; ref Hawkins

Appendix F. Lessons Learned Floursecent Microscopy

Because of their tough protein coats made of keratin, spores are highly resistant to normal staining procedures. The primary stain in the endospore stain procedure, malachite green, is driven into the cells with heat. Malachite green, also called aniline green, basic green 4, diamond green B, or victoria green B, IUPAC name:4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethyl-aniline is a toxic chemical primarily used as a dye. Since malachite green is water-soluble and does not adhere well to the cell, and since the vegetative cells have been disrupted by heat, the malachite green rinses easily from the vegetative cells, allowing them to readily take up the counterstain.

Most of the methods utilize heat to drive the stain into the endospore cell wall. One method, called the Schaeffer-Fulton Method, uses malachite green stain with heat and safranin for a counterstain. The endospore will stain green and the surrounding vegetative cell will stain pink. The heating step of the Schaeffer-Fulton Method was a concern due to impacts on the spores' susceptibility to future thermal treatment. There is a cold endospore staining method that utilizes malachite green by flooding the slide for at least 10-15 minutes and then counterstaining with safranin. The endospores will appear a lighter green than when heat is used. This method is safer and cleaner to use. Finally, the technique requires exposure of the spores to UV light during microscopy. Inactivation of the spores was observed due to either stain toxicity or UV exposure; or a combination of the two. Challenges with the technique included negative impacts on spores' susceptibility/viability from UV light and/or heating during staining, stain toxicity and loss of sample due to multiple washes included in the protocol. Technique

may be used in future work to determine population distributions, but concerns regarding sample loss may prevent its use. Staining is not recommended if spores are to be thermally treated and assessed for viability; even cold staining has suspected toxicity concerns. Under phase contrast observation at magnifications of 200x and 400x spores are readily observed in a concentrated (washed down) sample. Benefits of fluorescent microscopy do not outweigh the disadvantages.

Appendix G. Lessons Learned Sol-gel Application

Objective.

-Stabilize carbon population following application. Once wells were populated, carbon remained very unstable until some form of polymerization occurred.
-Function of this layer was to stabilize carbon as well as expel any trapped air interspersed between carbon particles.
Final protocol was determined through numerous trials. Potential causes that led to the failure of the initial runs and the resulting controls are summarized below:

Observations*:*

-*Problem*: Carbon dislodged during spin coating of silane.
-*Control*: Attempted to provide initial stabilization through vapor deposition with early organosilane selections (slower to spread). This method was employed due to concerns associated with dislodging the carbon from populated wells, however, vapor deposition proved to coat unevenly. Vapor deposition unneccesary and creates an uneven first coat which extrapolates problems with following coats. Carbon still not fixed to slide, can be removed using kim wipe so any stabilization it does provide is insufficient.

- Failed protocol: Apply thin coat of organosilane, Tetramethylorthosilicate, via vapor deposition following the first dry application of carbon. A vapor deposition system can be constructed using a large evaporation dish with some heat resistant object on which the slides can be placed atop. The organosilane is poured in to the bottom of the dish and the system is enclosed using a large watch glass over a hot plate; temperature less than vapor pressure of organosilane at approximately 80C.

Problem: Coatings at lower rpms resulted in rings around the wells; slower speed or more abundant debris resulted in larger rings.
Control: Less debris on glass surface and spin coating at higher speeds resulted in smaller rings or none at all. With increased speed the rings reduced.

Problem: Tetraethylorthosilicate was slow to spread and would flake upon executing a third application.
Control: Used a lower molecular weight silane, Dimethoxymethylvinylsilane, and two molar parts water. Follow on solutions had had less carbon groups and spread more quickly.

Problem: Carbon wells were depopulated by excsive speeds of spin coater or excessive silane. Organosilane being removed by centripetal forces may be responsible for depopulation, therefore, a slow rate of application/pipetting of the organosilane is required.
Control: Slowly pipet silane to the center of the slide while simultaneously initiating spin coater; delay of spin coater is sufficient to pipet entire quantity. Caution must be taken when spin coating slides. At rates greater than and equal to 1000rpm, higher molecular weight silanes will not spread quickly enough to avoid carbon depopulation due to

centripetal forces. Wells showed signs of depopulation and organosilane coating was not evident. Two coats at 4000rpm were sufficient. Observations indicated that organosilane spread quickly enough that carbon was not dislodged. Balance between even spreading; dice roll as to which occurs first-spread of silane or carbon particles being slung out of the well

Problem: Slides cannot be reused. Any attempt at carbon population following even the slightest silane application proved fruitless.
Control: None. Attempted alcohol and base washes under agitation, but pitting of the glass would occur prior to the removal of the silane from the wells.

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Vita

MAJ Bacon graduated from Norwich University-The Military College of Vermont in 1997 with a Bachelor of Science degree in Biology and Bachelor of Science in Environmental Science. In 2001 he graduated with a Master of Science in Engineering Management. Commissioned as a Second Lieutenant in the Corps of Engineers in 1997, MAJ Bacon served as a Platoon Leader and Battalion S4 during his first assignment at Fort Hood, Texas with the 299th Engineer Battalion-4th Infantry Division.

Reassigned to Bamberg, Germany as a member of the 1st Infantry Division, he served as an Assistant Operations Officer and Company Commander in the 82nd Engineer Battalion. MAJ Bacon has deployed to the Former Republic of Yugoslovia in support of OPERATION JOINT GUARDIAN (KFOR 4A) and most recently to Iraq in support of OPERATION IRAQI FREEDOM II (OIFII) returning this past spring.

MAJ Bacon's last assignment was with the U.S. Army Corps of Engineers, Philadelphia District where he served as Project Engineer and later as the Resident Engineer for the Delaware Resident Office located at Dover Air Force Base.

MAJ Bacon's next assignment will be with the Defense Threat Reduction Agency (DTRA) at Fort Belvoir, Virginia. There he will serve as the S3 Operations Officer for the Technical Support Division of the Combating Weapons of Mass Destruction Enterprise.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From – To)*
26-03-2009	Master's Thesis	March 2008 – March 2009

4. TITLE AND SUBTITLE

Thermal inactivation of *Bacillus anthracis* using laser irradiation of micro-etched platforms

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Bacon, Jeffrey B. Major, USA

5d. PROJECT NUMBER
If funded, enter ENR #

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES(S) AND ADDRESS(S)
Air Force Institute of Technology
Graduate School of Engineering and Management (AFIT/EN)
2950 Hobson Way, Building 640
WPAFB OH 45433-8865

8. PERFORMING ORGANIZATION REPORT NUMBER
AFIT/GWM/ENP/09-M01

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Ms. Angelica Rubio, Combating WMD Flight
709th Armament Systems Squadron
Kirtland Air Force Base, NM

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED.

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of this research was to determine the probability of kill for a thermal inactivation strategy for use against biological agents; specifically the resilient endospore of *Bacillus anthracis (Ba).* The effort focused on short durations (milliseconds to several seconds) and temperatures (300 to 1300 K) simulating the periphery effects after an explosion generated by conventional munitions. For an improved statistical counting, applied microlithography techniques were used to produce micro-etched glass platforms consisting of 532 circular sample wells, evenly spaced, with dimensions of 50μm in diameter and approximately 3μm in depth. Small carbon black radiators, which provide fast heating / cooling rate and confined temperature distribution, were produced by populating the etched wells with fine carbon particles of less than one μm for good contact with the spores. In order to prevent the carbon black from oxidation at high temperatures in air, a multifunctional sol-gel coating was designed to cover both the hydrophilic glass surface and hydrophobic carbon surface. Ba spores were sparely populated into the small wells on another micro-etched platform for improved statistical counting. The platform with carbon wells was paired with the other platform populated with the spores by aligning row by row and column by column using a laser diffraction method aided with an infrared beam finder. The study refined techniques to populate the sample wells with as few as one Ba spore per well. This enables researchers to qualify, quantify, treat and measure small samples of spores over time. Spores were heated against black carbon wells using a solid state laser (Nd: YAG). Heating temperatures were varied by using different laser powers. The heating times were controlled by adjusting the raster rate of the sample relative to the laser beam. The thermal radiation from the carbon well was measured using a Fourier-Transform Infrared (FTIR) spectrometer with a sensitive Mercury Cadmium Telluride (MCT) detector, from which the temperature was obtained by fitting a Planck function to the infrared spectrum from carbon. Samples were characterized before and after germination using optical microscopy and manual counting techniques. The statistical correlation between heating time, heating temperature, and spore viability was analyzed.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Larry Burggraf, Ph.D, AFIT
U	U	U	UU	117	19b. TELEPHONE NUMBER *(Include area code)* 785-3636 x4507 Larry.Burggraf@afit.af.mil

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39-18